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**PRESENCE OF INHIBIN AND ACTIVIN FAMILY OF
HORMONES AND THEIR INVOLVEMENT IN THE
REGULATION OF GONADOTROPIN RELEASE
IN THE GOLDFISH, *Carassius auratus***

by

Wei Ge



A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

Department of Zoology

Edmonton, Alberta

Fall 1993



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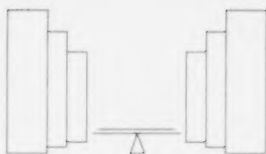
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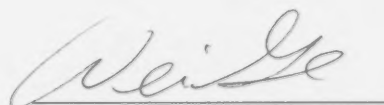
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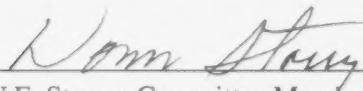
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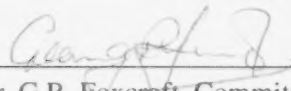
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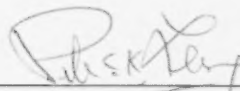
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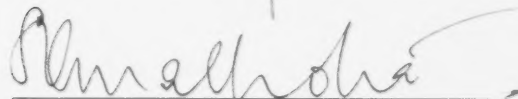
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Abstract

In mammals, inhibin and activin are dimeric proteins derived from different combinations of α , β A and β B subunits, with inhibin being $\alpha\beta$ A or $\alpha\beta$ B, and activin being β A β A, β A β B or β B β B. Inhibin preferentially suppresses FSH secretion, whereas activin is stimulatory to FSH.

It was found in the present study that aqueous crude goldfish ovarian extracts (CGOEs) have stimulatory effects on gonadotropin-II (GTH-II) release *in vitro* in goldfish. Interestingly, inhibin- and activin-containing porcine follicular fluid (pFF) was also stimulatory to goldfish GTH-II release; pFF and CGOEs are generally similar with regard to their actions on GTH-II release in goldfish. Consistent with the stimulatory actions of pFF, highly purified porcine inhibin A and activin A have stimulatory effects on GTH-II release in goldfish. The unique actions of inhibin were abolished by immunoneutralization with anti-inhibin antibodies. The actions of inhibin and activin are independent of GnRH, and the mechanisms mediating inhibin and activin actions may be different from those mediating GnRH. Using domain-specific antibodies, the inhibin and activin-like proteins were demonstrated immunocytochemically in goldfish gonads. To provide direct evidence for the existence of inhibin and activin family of proteins in goldfish, genes coding for inhibin and activin β A and β B subunits were cloned from goldfish genomic DNA. One form of β A and two forms of β B were obtained. Both goldfish β A and β B subunits show high structural conservation compared to mammalian counterparts. Southern analysis demonstrated at least two copies of genes for both β A and β B in the goldfish genome. Preliminary Northern analysis showed that goldfish activin β B subunit is expressed in the ovary and its expression significantly increases during recrudescence. In addition, activin and inhibin subunits, especially β A, have been localized immunocytochemically in the somatotrophs in the goldfish pituitary. Furthermore, porcine inhibin A and activin A were found to acutely stimulate goldfish GH secretion *in vitro*. These results indicate additional modes of action for inhibin and activin, i.e., paracrine and autocrine actions within the pituitary to regulate GTH and GH secretion.

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Abbreviations

Abbreviation	Description
bFF	bovine follicular fluid
CGOE	crude goldfish ovarian extract
DA	dopamine
E2	estradiol
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
5-HT	serotonin
FF	follicular fluid
FRP	FSH-releasing protein
FSH	follicle-stimulating hormone
FSP	FSH-suppressing protein
GABA	γ -aminobutyric acid
GH	growth hormone
GnRH	gonadotropin-releasing hormone
GnSIF	gonadotropin surge-inhibiting factor
GRF	growth hormone-releasing factor
GSi	gonadosomatic index (gonad wt/total body wt X 100)
GTH	gonadotropin
GTH-I	gonadotropin-I
GTH-II	gonadotropin-II
hCG	human chorionic gonadotropin
hF-GRP	human follicular gonadotropin-releasing peptide
HPLC	high performance liquid chromatography
IGF-I	insulin-like growth factor-I
LH	luteinizing hormone
MIF	mullerian inhibiting factor
NE	norepinephrine
NPY	neuropeptide Y
NTS	nucleus of solitary tract
pFF	porcine follicular fluid
PHA	phytohaemagglutinin
PVN	paraventricular nucleus
sGnRH	salmon gonadotropin-releasing hormone
SRIF	somatostatin
SON	supraoptic nucleus
T	testosterone
TGF- β	transforming growth factor β
TRH	thyroid hormone-releasing hormone
VIP	vasoactive intestinal peptide

Chapter One

GENERAL INTRODUCTION

In mammals, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are the two pituitary gonadotropic hormones that control gonadal steroidogenesis and gametogenesis in both sexes. Gonadotropin-releasing hormone (GnRH) is the major gonadotropin (GTH) stimulator from the hypothalamus which has actions on both LH and FSH release, and serves as a signal mediator from the central nervous system to the endocrine system (Conn *et al.*, 1987). The secretion of FSH and LH is also regulated indirectly by many other factors including neuropeptides such as neuropeptide Y (NPY) (McDonald *et al.*, 1985), and neurotransmitters such as norepinephrine (NE) and dopamine (DA) (Negro-Vilar *et al.*, 1979) that have influence on GnRH secretion. Gonadal steroids can also feed back either negatively or positively at the hypothalamic level to affect GnRH release, or at the pituitary level to influence FSH and LH production and the responsiveness of gonadotrophs to GnRH (Gharib *et al.*, 1990). The overall profiles of FSH and LH secretion are similar throughout the estrous cycle in various mammals, suggesting similar regulatory mechanisms for these two hormones. However, a variety of evidence also indicates that the release of FSH and LH may be differentially regulated. First, the levels of both FSH and LH increase after castration in rats (de Jong and Sharpe, 1976; Welschen *et al.*, 1979). The postcastration rise of FSH in rats can be effectively inhibited by administration of steroid-free bovine testicular extract and porcine follicular fluid (pFF) without affecting the increased LH levels (Rush and Lipner, 1979; Welschen *et al.*, 1979). Second, in the rodent estrus cycle, there is a preovulatory surge

release of both LH and FSH in the afternoon of proestrus; however, there is a secondary periovulatory surge of FSH in the early morning of estrus that is not accompanied by a concomitant surge of LH secretion (Chappel *et al.*, 1983). The second surge of FSH has been demonstrated to be independent of the actions of hypothalamic GnRH (Hasegawa *et al.*, 1981; Blake and Kelch, 1981; Schwartz *et al.*, 1985; Rush, 1985) and can be blocked by injection of pFF (Schwartz and Channing, 1977).

Gonadal steroid hormones had once been thought to be the only gonadal regulators to exert feedback actions on the production and release of GTHs from the anterior pituitary gland. However, evidence has accumulated during the past decades suggesting that gonads also produce certain nonsteroidal substance(s) of protein nature that regulate the production and secretion of GTHs in mammals. The most intensively studied of these proteins are inhibin and activin. Since both inhibin and activin show preferential actions on FSH secretion, these proteins have been considered to be among the factors contributing to the differential secretion of FSH and LH (for reviews see Vale *et al.*, 1988; Ying, 1988a, b). The research on inhibin and activin family has been progressing dramatically in mammals; however, very little attention has been paid to non-mammalian vertebrates.

Similar to tetrapods, two chemically distinctive GTHs have been identified and designated GTH-I and GTH-II in several teleostean species including chum salmon (Itoh *et al.*, 1988; Suzuki *et al.*, 1988a, b), coho salmon (Swanson *et al.*, 1991) and common carp (Van der Kraak *et al.*, 1992). However, unlike FSH and LH in mammals, there is no clear functional distinction between the two fish GTHs despite their structural similarities to mammalian GTHs (Suzuki *et al.*, 1988c; Van der Kraak *et al.*, 1992). Regarding neuroendocrine regulation, teleosts are somewhat unique in that the portal blood vessel system that serves as a connection delivering signals from the hypothalamus to the adenohypophysis is not functional; the pituitary in teleosts is directly

innervated by nerve terminals (for reviews see Peter *et al.*, 1986, 1990). In goldfish, the pituitary GTH-II is under a dual control of the hypothalamic GnRH and DA; GnRH is a potent GTH-II releasing factor, whereas DA has strong inhibitory actions on basal and GnRH-stimulated GTH-II secretion (Peter *et al.*, 1986). The release of GnRH in the hypothalamus and from the nerve endings within the pituitary is subject to the influence of various factors including DA, serotonin (5-HT) and NE (Yu *et al.*, 1991). In addition to GnRH and DA, many other factors have been implicated in the modulation of GTH-II release either directly at the pituitary level or indirectly via hypothalamic GnRH or DA. These include hypothalamic neuropeptides such as NPY (Peng *et al.*, 1990, 1993) and opiates (Rosenblum and Peter, 1989), and neurotransmitters such as NE (Chang *et al.*, 1991) and 5-HT (Somoza and Peter, 1991). These factors are all stimulatory to pituitary GTH-II release. γ -aminobutyric acid (GABA) and taurine also have stimulatory effects on GTH-II release and their actions may be mediated through the DA system (Sloley *et al.*, 1992). Gonadal steroids play important roles in feeding back to influence GTH-II production and secretion, and both negative and positive actions of steroids have been demonstrated. Kobayashi and Stacey (1990) demonstrated a sharp increase of GTH-II levels in goldfish plasma after gonadectomy. The elevated GTH-II levels could be brought down to normal by implantation of exogenous steroids (testosterone, T and estradiol, E2), suggesting a negative feedback by gonadal steroids in maintaining basal GTH-II release. On the other hand, Trudeau *et al.* (1991, 1993) found that implantation of solid silastic pellets containing T and E2 in the intact goldfish had no effects on basal GTH-II levels in the plasma, but could potentiate the GTH-II response to exogenous GnRH challenges. Unfortunately, although gonadal peptide factors such as inhibin and activin have been established as important GTH regulators that play critical roles during the reproductive cycle in mammals, no information is available on the existence and involvement of gonadal peptides in the regulation of GTH release in teleosts.

Inhibin and Activin Family of Hormones

Inhibin and activin are members of transforming growth factor β (TGF- β) superfamily, consisting of inhibin, activin, TGF- β (Derynck *et al.*, 1985; Sporn *et al.*, 1986), Mullerian inhibiting factor (MIF) (Cate *et al.*, 1986), a decapentaplegic gene complex in *Drosophila* (Padgett *et al.*, 1987), VG1 gene of *Xenopus* (Weeks and Melton, 1987) and the bone morphogenetic proteins BMP-2A and BMP-3 (Wozney *et al.* 1989). Among the members of the family, only inhibin and activin have been demonstrated to be involved in the regulation of pituitary GTH secretion.

Brief History

The term "inhibin" was originally proposed by McCullagh in 1932 to designate a putative factor in the aqueous extracts of bull testes which could suppress the appearance of "castration cells" in the anterior pituitary gland. After FSH and LH were differentiated, the putative inhibin had been demonstrated to have specific inhibitory effects on FSH secretion. However, the research on inhibin had been hampered for more than four decades because of the lack of a proper purification technique and an appropriate bioassay to monitor the purification process. During the past fifteen years, inhibin activity of suppressing basal FSH release has been demonstrated in various gonadal fluids and extracts including ovarian follicular fluids (FFs) (Chari *et al.*, 1979; de Jong and Sharpe, 1976; Fujii *et al.*, 1983; Kling *et al.*, 1984; Koiter *et al.*, 1983; Miller *et al.*, 1979; Summerville and Schwartz, 1981; Williams and Lipner, 1981),

ovarian extracts (Channing *et al.*, 1985; Chappel, 1979; Torney *et al.*, 1991), rete testis fluids (Bardin *et al.*, 1987; Setchell and Jacks, 1974; Setchell and Sirinathsinghji, 1972), testicular extracts (Koegh *et al.*, 1976; Murthy *et al.*, 1979; Sheth *et al.*, 1979; Torney *et al.*, 1991) and seminal plasma (Chari *et al.*, 1978; Sheth *et al.*, 1984) from a variety of mammalian species including pigs (Lumpkin *et al.*, 1984; Marder *et al.*, 1977; Summerville and Schwartz, 1981; Williams and Lipner, 1981), cows (Chari *et al.*, 1978; de Jong and Sharpe, 1976; Koegh *et al.*, 1976; Koiter *et al.*, 1983), sheep (Bardin *et al.*, 1987; Murthy *et al.*, 1979; Sheth *et al.*, 1979), rats (Fujii *et al.*, 1983), hamsters (Chappel, 1979), horses (Miller *et al.*, 1979) and human beings (Chari *et al.*, 1979; Sheth *et al.*, 1984). Meanwhile, considerable research effort had been invested in a search for the putative inhibin molecules. In 1985, armed with high performance liquid chromatography (HPLC) and a sensitive bioassay of inhibition of basal FSH release by dispersed rat pituitary cells in static culture, four laboratories simultaneously reported purification of inhibin molecules from porcine (Ling *et al.*, 1985; Miyamoto *et al.*, 1985; Rivier *et al.*, 1985) and bovine follicular fluid (bFF) (Robertson *et al.*, 1985). Interestingly, during the course of inhibin purification from pFF, another novel protein was identified and purified. In contrast to inhibin, this protein specifically stimulates FSH release from cultured rat pituitary cells and was named FSH releasing protein (FRP) (Vale *et al.*, 1986) or activin (Ling *et al.*, 1986). Biochemical characterization revealed that activin is structurally related to inhibin (Ling *et al.*, 1986; Vale *et al.*, 1986).

Chemical Characteristics

Purification of inhibin and activin from natural sources from a variety of mammalian species and subsequent cloning of their genes have greatly enhanced our understanding of molecular structures of this family of proteins. Two forms of inhibin

with molecular weights of 32 kD and 58 kD have been reported in murine, ovine, bovine, porcine and human testes or ovaries (Bicsak *et al.*, 1988; de Jong, 1988; Forage *et al.*, 1986; Li *et al.*, 1985; McNeilly *et al.*, 1988; Robertson *et al.*, 1985; Ying, 1988a, 1988b). The 32 kD form is generally believed to be the mature biologically active inhibin, whereas the 58 kD form may be the precursor that is somehow enzymatically processed into the mature form. The 32 kD form of inhibin is a glycosylated heterodimer consisting of two distinctive subunits, a 18 kD α subunit and a 14 kD β subunit, which are linked together by a disulfide bridge. The α subunit contains one or two glycosylation sites, whereas the β subunits seem to lack glycosylation sites (de Jong *et al.*, 1982; Ling *et al.*, 1985; Mason *et al.*, 1985; Rivier *et al.*, 1985; Vale *et al.*, 1988). Two types of inhibin (inhibin A and inhibin B) have been found in porcine (Ling *et al.*, 1985; Mason *et al.*, 1985), human (Mason *et al.*, 1986) and murine follicular fluid (Esch *et al.*, 1987; Woodruff *et al.*, 1987). Inhibin A and inhibin B share an identical α -subunit, but each has a distinct but related β -subunit, designated β A and β B, with 70% homology. Activin is structurally related to inhibin in that it is a homodimer of inhibin β subunits (Ling *et al.*, 1986; Rivier *et al.*, 1987a; Vale *et al.*, 1986). Two types of activin have been identified in pFF and named activin AB (β A β B) and activin A (β A β A), respectively (Ling *et al.*, 1986; Vale *et al.*, 1986; Ying, 1988b). Although the putative activin B (β B β B) has not been purified from natural sources so far, its existence has been indirectly demonstrated by immunoneutralization with a specific monoclonal antibody against activin B (Corrigan *et al.*, 1991). The genomic and molecular structures of inhibin and activin and their relations are depicted in Fig. 1.1.

Human, porcine, bovine, ovine and murine inhibin and activin subunits have been shown to be highly conserved. β A and β B subunits from these species show more than 95% homology at the amino acid level, whereas the α subunit exhibits more variation with 80% homology among these species (Esch *et al.*, 1987; Forage *et al.*,

1986; Mason *et al.*, 1985, 1986; Mayo *et al.*, 1986; Rivier *et al.*, 1987a; Stewart *et al.*, 1986; Vale *et al.*, 1988; Ying, 1988a, 1988b). Such a high structural conservation suggests that inhibin and activin must play critical physiological roles in mammals.

Production Sites

Gonadectomy reduces serum inhibin to undetectable levels in male and female rats (Robertson *et al.*, 1988a), indicating that the gonads are the primary sources of circulating inhibin. Cell culture experiments and immunohistochemical studies have shown that ovarian granulosa cells and testicular Sertoli cells are the major sites of inhibin and activin production in female and male gonads, respectively (Cuevas, 1987; de Jong, 1988; de Winter *et al.*, 1992; Eramaa *et al.*, 1993; Merchenthaler *et al.*, 1987; Morris *et al.*, 1988; Zhang *et al.*, 1987). *In vitro* perfusion of individual porcine follicles shows that the mature dominant follicles may be the principal source of inhibin production during the ovarian cycle (Westhof *et al.*, 1990). Although some experiments show that purified Leydig cells from rats also produce immunoactive and bioactive inhibin *in vitro* (Risbridger *et al.*, 1989), it is generally believed that Leydig cells are not the major source of testicular inhibin under normal physiological conditions (de Kretser *et al.*, 1989; de Winter *et al.*, 1992).

Nucleic acid hybridization and immunohistochemistry show that in addition to the gonads, inhibin and activin subunits are also expressed in a variety of extragonadal tissues, including the brain, pituitary, placenta, adrenal, kidney, spleen and bone marrow (Meunier *et al.*, 1988; Crawford *et al.*, 1987; Roberts *et al.*, 1989). Such a diverse distribution of inhibin and activin subunits suggests multiple biological functions for inhibin and activin, and points to the possibility that inhibin and activin may play widespread paracrine and/or autocrine roles in different tissues. The most interesting

finding of these studies is the expression of inhibin and activin subunits within the pituitary, the major target organ of inhibin and activin actions studied so far. The immunoreactive α and β B subunits in the rat pituitary have been demonstrated in both FSH and LH cells (Roberts *et al.*, 1989). Inhibin and activin subunits have also been reported in the growth hormone (GH) cell line GH3 (Li *et al.*, 1992). These findings suggest that inhibin and activin may play paracrine or autocrine roles within the pituitary to regulate the secretion of GTH and perhaps also other pituitary hormones.

Biological actions

Although both inhibin and activin were originally identified as specific pituitary FSH regulators, and their actions on GTH release are still the most extensively studied aspect of their functions, evidence has accumulated showing that inhibin and activin, particularly activin, are also involved in many other physiological processes. Interestingly, activin and inhibin often functionally antagonize each other in most, if not all, cases (for review see Vale *et al.*, 1988).

Actions in pituitary Gonadal proteins, especially inhibin, have been thought to be involved in the differential regulation of FSH release (Woodruff and Mayo, 1990). It is now well documented and accepted that gonadal inhibin from either ovarian or testicular origin preferentially suppresses basal and GnRH-stimulated FSH production both *in vivo* and *in vitro* (Bicsak *et al.*, 1986; 1987; Campen and Vale, 1988; Erickson and Hsueh, 1978; Lee *et al.*, 1981; Morris *et al.*, 1988; Rivier *et al.*, 1988, 1991; Zhang *et al.*, 1987). Inhibin-binding sites have been demonstrated in the rat anterior pituitary and appear to be distinct from GnRH-binding sites (Seethalakshmi *et al.*, 1984). The physiological role played by inhibin in regulating FSH release has been confirmed *in vivo* by passive immunization. Injection of specific anti-inhibin antibodies

produced a significant elevation in plasma FSH levels in female (Culler and Negro-Vilar, 1988; Rivier *et al.*, 1986, 1987b) and male rats (Culler and Negro-Vilar, 1988; Rivier *et al.*, 1988). Results from these studies also suggest sex-related differences with regard to inhibin regulation of FSH secretion (Culler and Negro-Vilar, 1988). Inhibin seems to play significant roles controlling FSH release in peripubertal and adult female rats; however, in the male, the physiological roles played by endogenous inhibin with regard to FSH secretion appear mainly restricted to the prepubertal stage (Culler and Negro-Vilar, 1988; Rivier *et al.*, 1986, 1987b, 1988). One possible reason for this disparity may be that unlike adult males whose plasma levels of FSH usually remain stable, serum FSH levels in the female exhibit significant fluctuations during reproductive cycles, necessitating more complex regulatory mechanisms.

To date most detailed studies regarding inhibin actions on GTH release have been based on an *in vitro* bioassay utilizing static cultures of dispersed rat pituitary cells. Since actions of inhibin are usually slow, most *in vitro* studies employ a protocol of long term incubation in which the dispersed pituitary cells are exposed to inhibin or inhibin preparations for at least 24 hours, mostly 72 hours (for reviews see Vale *et al.*, 1988; Ying 1988a, b). Under nonstimulated conditions in rat pituitary cell culture, inhibin exerts its inhibitory actions on basal FSH secretion by directly suppressing the mRNA expression and biosynthesis of FSH (Attardi *et al.*, 1989a; Carroll *et al.*, 1989; de Jong *et al.*, 1982; de Jong, 1988; Erickson and Hsueh, 1978; Mercer *et al.*, 1987; Scott and Burger, 1981; Vale *et al.*, 1988) and enhancing the decay rate of FSH mRNA (Attardi and Winter, 1993; Farnworth *et al.*, 1988a), resulting in a lower intracellular concentration of GTHs. The effect of inhibin on the decay rate of FSH mRNA is thought to be mediated by inducing the transcription of a protein factor that promotes the degradation of FSH mRNA (Attardi and Winter, 1993).

It is commonly accepted that inhibin has little effect on basal LH secretion despite

occasional reports that inhibin also suppresses LH β mRNA levels, inhibits basal LH release and increases intracellular degradation of LH in the rat under certain experimental conditions (Attardi *et al.*, 1989b; Farnworth *et al.*, 1988a; Jakubowiak *et al.*, 1991). However, it is well documented that inhibin suppresses the GnRH-stimulated release of FSH and LH both *in vitro* and *in vivo* (Campen and Vale, 1988; Culler and Negro-Vilar, 1989; Farnworth *et al.*, 1988b; Jakubowiak *et al.*, 1989, 1990, 1991; Kotsuji *et al.* 1988; Vale *et al.*, 1986; Ying, 1988a, 1988b). Therefore, besides direct actions on basal FSH release, inhibin also regulates secretion of both FSH and LH by indirectly influencing GnRH actions. The inhibitory effects of inhibin on GnRH-stimulated GTH secretion are, at least partly, due to a dramatic decrease in GnRH receptor number on gonadotrophs; however, the binding affinity of GnRH receptors is not affected by inhibin treatment (Attardi *et al.*, 1989b; Wang *et al.*, 1988). Furthermore, inhibin also suppresses GnRH self-priming (Culler, 1992), which is probably due to the inhibition of GnRH-induced up-regulation of GnRH receptors (Wang *et al.*, 1989).

The mechanisms whereby inhibin selectively suppresses basal FSH but not LH secretion in rats remains unknown. One possibility may be related to the different dynamics of FSH and LH biosynthesis and secretion. Under unstimulated culture conditions, FSH biosynthesis and secretion remain high and autonomous whereas LH biosynthesis shows greater dependence on GnRH stimulation (Franchimont *et al.*, 1986). FSH is stored inside the cell for a shorter time than LH, resulting in a smaller intracellular pool of FSH; therefore, the basal release of FSH is directly related to the synthesis rate of the hormone (Farnworth *et al.*, 1988b). Inhibin has direct inhibitory actions on FSH gene expression, exerting significant effects on the basal FSH release that is more dependent on the rate of biosynthesis than is the case for LH. The occasionally reported effects of inhibin on basal LH release may be due to other mechanisms that do not involve hormone synthesis, such as increased intracellular

degradation (Farnworth *et al.*, 1988b), which, together with the reduction of GnRH receptor number, also contributes to the inhibition of GnRH-stimulated secretion of LH due to the reduced intracellular releasable pools.

In sharp contrast to its actions in the rat, inhibin shows opposite actions in ovine pituitary cell culture in terms of its effects on GnRH receptor and GnRH-stimulated LH secretion. Porcine and bovine inhibin have been demonstrated to increase rather than decrease GnRH receptor number in cultured ovine pituitary cells (Gregg *et al.*, 1991; Laws *et al.*, 1990). In agreement with this, GnRH-stimulated ovine LH release is significantly potentiated by either bFF or highly-purified inhibin in a dose- and time-dependent manner, although both preparations, as in rats, inhibit basal and GnRH-stimulated FSH secretion (Gregg *et al.*, 1991; Muttukrishna and Knight, 1990). This unique stimulatory action can be blocked by specific anti-inhibin serum (Muttukrishna and Knight, 1990). These *in vitro* findings also provide an explanation for the previously puzzling *in vivo* observations that administration of inhibin-containing preparations to ewes lowered plasma FSH levels but dramatically increased LH release (Knight and Gastillo, 1988; Wallace and McNeilly, 1986) and potentiated the LH response to exogenous GnRH challenges (Miller and Huang, 1985). Taken together, these results demonstrate two important points. First, the differential regulation of FSH and LH may be realized by use of a variety of mechanisms in different species. Second, inhibin is not always inhibitory in terms of its action on GTH release, as generally accepted and as its name implies. Inhibin of the same source may have, depending on species, either stimulatory or inhibitory actions on GTH secretion directly, or indirectly through modulation of GnRH actions. Undoubtedly, much more work needs to be done to elucidate the species specificity of inhibin actions.

The research on actions of activin on GTH release has been basically restricted to *in vitro* studies using rat pituitary cell cultures. Activin stimulates the basal secretion of

FSH and increases FSH β mRNA expression by cultured rat pituitary cells (Carroll *et al.*, 1989; Ling *et al.*, 1986; Vale *et al.*, 1986; Ying, 1988a). It also stabilizes FSH β mRNA (Carroll *et al.*, 1991). The stimulatory effects of activin on basal FSH secretion are different from those of hypothalamic GnRH which exhibits its effects within seconds of application. The maximal effects of activin on FSH secretion are usually observed after 24-48 hour incubation (for reviews see Ying 1988a, 1988b). Since the GnRH-desensitized pituitary cells respond to activin treatment and GnRH antagonists do not affect the activities of activin, it appears that the mechanisms of action of activin are distinct from those that mediate the actions of GnRH (Risbridge *et al.*, 1990; Vale *et al.*, 1986). In contrast to GnRH, the actions of which tend to reduce intracellular FSH levels, activin-treated cells have increased intracellular FSH contents, suggesting a higher ability for activin to stimulate biosynthesis of FSH (Ying, 1988a). Limited *in vivo* studies showed that administration of natural or recombinant activin significantly increased plasma FSH levels in immature female rats (Doi *et al.*, 1992; Schwall *et al.*, 1989) and adult male macaques (McLachlan *et al.*, 1989), and potentiated exogenous GnRH-stimulated FSH and LH release in the macaque (McLachlan *et al.*, 1989).

One interesting aspect of *in vitro* studies on inhibin and activin that has attracted some attention recently is the significant disparity between different *in vitro* systems, static culture vs. perfusion of dispersed pituitary cells, regarding inhibin and activin actions. The majority of research has been carried out using static cultures of plated pituitary cells. Recent studies using pituitary cell perfusion have provided some new information on the actions of inhibin and activin on GTH release which is distinct from that obtained from static cell cultures. In pituitary cell perfusion, activin stimulates FSH β mRNA expression within a much shorter period of time but with much higher potency than in static culture of dispersed cells (75-fold after 6 hour treatment vs. 2.7-fold after 72 hour treatment) (Weiss *et al.*, 1992). In static culture, activin and inhibin usually

have little effects on basal LH secretion; however, there is a quick release of both FSH and LH in response to activin, with a peak reached during the first hour of perfusion, and 8 hour treatment with inhibin causes 30-40% and 50-60% inhibition of FSH and LH secretion, respectively (Weiss *et al.*, 1992). Generally, the time needed for inhibin and activin to reach their maximal effects in perfusion is significantly shortened compared to that in static culture (Kotsuji *et al.*, 1988; Jakubowiak *et al.*, 1989, 1990, 1991; Weiss *et al.*, 1992). One of the reasons for these differences may be that in the static culture there is an accumulation of pituitary cell-derived regulatory factors that interfere with the actions of exogenous secretagogues. These factors are immediately carried away from pituitary cells by the medium in perfusion (Weiss *et al.*, 1992).

In addition to regulation of GTH secretion, inhibin and activin, especially activin, have also been shown to exert direct actions on other types of pituitary cells, particularly somatotrophs. Activin inhibits basal and growth hormone-releasing factor (GRF)-stimulated GH secretion in static cultures of dispersed rat pituitary cells (Bilezikjian *et al.*, 1990; Billestrup *et al.*, 1990), and completely blocks the GH response to thyroid hormone-releasing hormone (TRH) in cultured human GH-secreting tumour cells (Kitaoka *et al.*, 1991). Thirty minute and 3 hour treatments are required to see minimum and maximum effects of activin, respectively. The mechanisms of the inhibitory action of activin on GH remain unknown. Activin alone does not affect intracellular cAMP and calcium levels, but it reduces GRF-stimulated cAMP production (Bilezikjian *et al.*, 1990) and TRH-stimulated increases in intracellular calcium levels (Kitaoka *et al.*, 1991). Activin and somatostatin (SRIF), a chief inhibitor of GH secretion, show additive actions on GH release. Treatment of pituitary cells with activin also suppresses GH responses to other pharmacological agents such as 12-O-tetradecanoyl-phorbol-13-acetate, forskolin, cholera toxin, and 8-bromo-cAMP. Pertussis toxin can partially reverse the inhibitory effects of activin on GH secretion, suggesting that Gi protein may

be involved in the signal transduction of activin (Bilezikjian *et al.*, 1990).

Although activin has been generally accepted as a gonadal protein and its actions on pituitary GTH secretion have been extensively studied, it has been unknown, due to the lack of specific assays for measurement of concentrations of activin, whether this protein is actually released as a hormone from gonads as originally believed, or if it mainly functions as a local paracrine and/or autocrine regulator. A variety of evidence has emerged pointing to the latter possibility. Activin subunit mRNAs are widely expressed in various tissues such as ovary, testis, placenta, pituitary, adrenal, bone marrow, spleen, brain and spinal cord (Meunier *et al.*, 1988) most of which have been demonstrated to be the targets of activin actions. The immunoreactive activin subunits are also present within the rat pituitary (Roberts *et al.*, 1989). These subunits have been shown immunocytochemically to be produced by FSH and LH cells in the rat (Roberts *et al.*, 1989). Using a specific radioimmunoassay, Shintani *et al.* (1991) also demonstrated a high level of activin A in rat pituitary. The GH3 cell line also expresses activin subunits *in vitro*, implying that GH cells could also be a site for activin production (Li *et al.*, 1992). In addition, specific anti-activin B monoclonal antibody can dramatically suppress the basal release of FSH by cultured rat pituitary cells in a dose-dependent manner, suggesting that a critical role for pituitary activin B is to regulate the basal levels of FSH secretion *in vitro* (Corrigan *et al.*, 1991); therefore, the "spontaneous" secretion of FSH *in vitro* is, at least to some extent, activin-stimulated release. Recently, specific enzyme-linked immunosorbent assays (ELISAs) for activin A and activin B have been developed using monoclonal antibodies and used to study the cyclic changes in plasma activin levels throughout menstrual cycles in women (Wong *et al.*, 1992). Surprisingly, plasma activin concentrations remain unchanged and almost undetectable throughout the menstrual cycle. Based on these lines of evidence, it is now generally believed that activin plays trivial roles as an endocrine hormone, but functions

as an important paracrine and/or autocrine regulator in the tissues where it is produced (Chen, 1993).

The concept of the intrinsic paracrine and/or autocrine regulation of GTH secretion by pituitary-derived activin is further supported by *in vivo* studies. Ovariectomy was found to enhance the expression of activin β B subunit in rat pituitary and the enhancement can be prevented by estrogen replacement (Roberts *et al.*, 1989). In agreement with this, ovariectomy causes a hypersecretion of FSH from the allografted rat pituitary gland (DePaolo, 1991). This post-ovariectomy hypersecretion of FSH from the allografted pituitary can be significantly suppressed by passive immunoneutralization with a specific monoclonal anti-activin B antibody, strongly suggesting that a rise in pituitary activin production contributes to the post-ovariectomy hypersecretion of FSH (DePaolo *et al.*, 1992). Interestingly, injection of the same antibody into the intact rat also partially suppresses the typical secondary periovulatory surge of FSH at estrus, indicating that pituitary activin may also play important physiological roles in the induction of the secondary surge of FSH in the rat ovarian cycle (DePaolo *et al.*, 1992). Ovarian inhibin is also important in the generation of the secondary surge of FSH since the plasma inhibin levels decline abruptly right after the primary FSH surge and at the beginning of the the second periovulatory FSH surge (Haisenleder *et al.*, 1990; Rivier and Vale, 1989). Taken together, these lines of evidence point to a new notion that the control of pituitary GTH secretion, at least FSH, involves regulatory signals from the central nervous system, peripheral gonads, and from within the pituitary itself through endocrine, paracrine or autocrine pathways. That gonadal steroids may influence the production of pituitary activin, which in turn affects the secretory activity of FSH cells, provides a new mechanism for the feedback actions of gonadal steroids on GTH secretion.

Inhibin may also act as a paracrine and/or autocrine regulator since inhibin-

specific α subunit has been found in a variety of extragonadal tissues (Meunier *et al.*, 1988). However, compelling evidence demonstrates that inhibin is also working as an endocrine hormone. The plasma has substantial levels of inhibin (Campbell *et al.*, 1990; Hasegawa *et al.*, 1988; Rivier and Vale, 1987b; Robertson *et al.*, 1988b) which fluctuate during the reproductive cycle (Campbell *et al.*, 1990; Findlay *et al.*, 1990; Haisenleder *et al.*, 1990; Hasegawa *et al.*, 1988; Rivier and Vale, 1987b; Robertson *et al.*, 1988b) and show an overall inverse relationship with FSH levels (Findlay *et al.*, 1990; Haisenleder *et al.*, 1990). Immunoneutralization of endogenous inhibin significantly increased the plasma concentrations of FSH (Rivier and Vale, 1987b; Culler and Negro-Vilar, 1988) and FSH β subunit mRNA expression in the pituitary (Attardi and Vale, 1992). Substantial amounts of inhibin have been demonstrated to be released into the spermatid vein blood in a pulsatile manner (Winter, 1990), and gonadectomy dramatically reduces the plasma levels of inhibin (Abeyawardene *et al.*, 1989; Findlay *et al.*, 1990; Hasegawa, Y. *et al.*, 1988; Robertson *et al.*, 1988a) with a concomitant increase in plasma FSH concentrations (Medhamurthy *et al.*, 1990). This post-gonadectomy rise in FSH levels can only be prevented by administration of both inhibin and steroids (Martin *et al.*, 1989). Hypophysectomy also causes a decline in plasma inhibin levels which can be restored with FSH administration (Au *et al.*, 1985), confirming a closed-loop endocrine feedback between FSH and inhibin.

Actions in gonads In addition to regulating GTH secretion from the pituitary gland, gonadal inhibin and activin also function as intragonadal paracrine or autocrine regulators to influence gonadal steroidogenesis and gametogenesis.

Binding sites for both inhibin and activin have been demonstrated on rat ovarian granulosa cells (Woodruff *et al.*, 1990). Inhibin alone does not appear to affect gonadal steroid production; however, inhibin inhibits FSH-stimulated E₂ (Ying *et al.*, 1986) and progesterone (Hutchinson *et al.*, 1987) secretion by rat ovarian granulosa cells *in vitro*.

By contrast, LH-stimulated androgen production by rat testicular Leydig cells and ovarian theca cells is enhanced by inhibin (Hsueh *et al.*, 1987). Similarly, activin alone does not have effects on gonadal steroidogenesis; however, in contrast to inhibin, activin enhances FSH-induced estrogen production by cultured granulosa cells (Vale *et al.*, 1988; Ying *et al.*, 1986) but suppresses LH or human chorionic gonadotropin (hCG)-induced androgen production by rat testicular Leydig cells and ovarian theca cells (Hsueh *et al.*, 1987; Lin *et al.*, 1989). LH-stimulated cAMP production by rat testicular Leydig cells is attenuated by activin (Lin *et al.*, 1989). Activin also stimulates ovarian inhibin expression and production (Doi *et al.*, 1992; LaPolt *et al.*, 1989; Sugino *et al.*, 1988).

One interesting aspect of activin actions within the ovary is its possible involvement in the induction of follicular atresia. Unilateral injection of recombinant human activin into the ovarian intrabursal space causes follicular atresia, suggesting that ovarian activin may act locally to induce atresia (Woodruff *et al.*, 1990). By contrast, injection of inhibin promotes follicular growth.

Inhibin and activin also act directly on germ cells to affect gametogenesis in both males and females. Binding sites for inhibin and activin have been demonstrated on rat testicular germ cells especially spermatogonia (Woodruff *et al.*, 1992). Inhibin suppresses the DNA synthesis in spermatogonia (Franchimont *et al.*, 1981) and spermatogonial proliferation (van Dissel-Emiliani *et al.*, 1989). It also inhibits oocyte meiotic division in rats (O *et al.*, 1989). Activin, however, stimulates rat spermatogonial proliferation (Mather, *et al.*, 1990). The germ cells may in turn exert paracrine influences on inhibin and activin production since their subunit and mRNA levels in testis are stage-dependent (Bhasin *et al.*, 1989; Kaipia *et al.* 1991).

Actions in nervous system All three inhibin and activin subunit mRNAs (α , βA and βB) have been shown to be expressed in rat brain and spinal cord (Meunier *et al.*, 1988), suggesting that these proteins may have regulatory roles in the nervous

system. Immunoreactivities for β A and β B, but not α , subunits have been demonstrated in a group of neurons situated in the nucleus of the solitary tract (NTS), a major recipient of visceral physiological inputs (Sawchenko *et al.*, 1988). The immunoreactive nerve terminals have been found in the septal-preoptic region where neurons containing GnRH are located. The immunoreactive fibres also exist in a pathway that projects synaptically to oxytocinergic neurons in the paraventricular (PVN) and supraoptic (SON) nuclei, and the immunoreactivities with anti- β A and β B have been further demonstrated ultrastructurally in the secretory vesicles within presynaptic terminals in the PVN and SON (Sawchenko *et al.*, 1988). These findings are in agreement of the biological actions of inhibin and activin in the brain. Inhibin appears to exert an inhibitory action on rat hypothalamic GnRH production and secretion (de Greef *et al.*, 1983; Demoulin *et al.*, 1979). Injection of activin into the PVN stimulates oxytocin secretion in the rat, and infusion of anti- β sera in the PVN suppresses suckling-induced oxytocin release, suggesting that activin may be involved in the mediation of suckling-induced oxytocin secretion (Sawchenko *et al.*, 1988).

Activin has also been demonstrated to be a nerve cell survival factor *in vitro* (Schubert *et al.*, 1990). Activin A promotes the survival of the P19 nerve-like cell line, neural retina, and the B50 nerve cell line derived from the rat CNS in serum-free medium. Interestingly, inhibin also promotes the survival of the B50 nerve cell line, providing an example that activin and inhibin do not antagonize one another like in most other systems.

Actions in placenta Inhibin-like activity was demonstrated in the extracts of rabbit placenta (Hochberg *et al.*, 1981), and inhibin subunit mRNAs are expressed in the rat and human placenta (McLachlan *et al.*, 1986; Meunier *et al.*, 1988), suggesting potential roles by these proteins in the regulation of placental functions. Activin treatment for 48 hours significantly increases GnRH and progesterone secretion by cultured

human placenta cells (Petraglia *et al.*, 1989). Although activin alone causes a slight increase in hCG release, it dramatically potentiates GnRH-stimulated hCG secretion. Inhibin alone does not exert any actions on GnRH, hCG and progesterone secretion; however, it potently reverses the actions of GnRH and activin (Petraglia *et al.*, 1989).

Actions in liver Activin has potent and acute stimulatory effects on glucose release by cultured rat hepatocytes (Mine *et al.*, 1990). Eleven minute treatment with activin causes a rapid release of hepatic glucose. The maximal effect of activin (10^{-9}M) is about 50% of that of glucagon, and the actions of these two glucose releasers are additive. Activin does not increase cellular cAMP concentration, but induces a rapid intracellular calcium mobilization. Consistently, 20 sec treatment with activin causes a very rapid inositol trisphosphate production. These studies provide an example for a very acute effect of activin and suggest one possible second messenger pathway mediating activin actions in mammals.

Actions in pancreatic islets In static cultures of rat pancreatic islets, activin stimulates insulin secretion in a dose dependent manner. Furthermore, activin treatment greatly potentiates glucose-induced insulin release. In a perfusion system, however, activin alone does not seem to affect basal insulin release, but it synergistically potentiates glucose-induced insulin secretion. The action of activin on insulin secretion is greater in the perfusion system than in the static culture system (Totsuka *et al.*, 1988).

Actions in bone marrow A protein factor called erythroid differentiation factor, EDF, was purified from conditioned medium of the leukaemia cell line THP-1, and characterized to be activin (Eto *et al.*, 1987). Since then, some studies have been carried out on the involvement of the inhibin and activin family in the regulation of blood cell growth and differentiation. Treatment of K562 cells, a human erythroleukaemic cell line, with activin dramatically increases the cellular content of haemoglobin, whereas addition of inhibin suppresses the action of activin. In human bone marrow cultures,

activin stimulates and inhibin inhibits erythroid colony formation, respectively, in the presence of erythropoietin (Yu *et al.*, 1987), suggesting these two proteins are involved but functionally antagonistic in the modulation of proliferation and differentiation of erythroid progenitor cells. In agreement with these *in vitro* results, *in vivo* administration of recombinant human activin A causes a significant increase in the circulating concentrations of red blood cells and hemoglobin in rats (Schwall *et al.*, 1989). These lines of evidence, together with the finding that activin β A subunit mRNA is expressed in rat bone marrow (Meunier *et al.*, 1988), support the possibility that activin, and probably inhibin as well, may play paracrine and/or autocrine roles in normal erythroid differentiation.

Actions in thymus Inhibin and activin are also involved in immunoregulation. Thymidine incorporation by cultured adult rat thymocytes, in the presence of phytohaemagglutinin (PHA), is stimulated by bovine inhibin, and inhibited by bovine activin (Hedger *et al.*, 1989). Expression of inhibin and activin subunits in other immune system tissues such as bone marrow and spleen (Meunier *et al.*, 1988) also suggests potential roles for inhibin and activin in immunoregulation.

Actions in embryonic development A highly potent mesoderm-inducing factor recently purified, sequenced and cloned from *Xenopus* (Asashima *et al.*, 1991a; Smith *et al.*, 1990; Thomsen *et al.*, 1990; van den Eijnden *et al.*, 1990), chicken (Asashima *et al.*, 1991b; Mitrani *et al.*, 1990) and the murine cell line WEHI-3 (Albano *et al.*, 1990) turned out to be activin, that is expressed early during embryogenesis in *Xenopus* and chick. Activin has the highest potency of factors identified so far in inducing mesoderm formation during the development. One exciting aspect of activin action is that this protein is very versatile in that it can induce formation of all kinds of mesodermal organs, depending on the concentrations (Asashima *et al.*, 1991b).

To summarize, inhibin and activin family of hormones, particularly activin, have

multiple functions in such fundamental biological processes as reproduction, development, cell proliferation and differentiation. Based on the information described above, it is very clear that the cellular and molecular mechanisms mediating the diverse functions of these proteins in different tissues are also diverse. For example, activin activates the calcium messenger system in some cell types such as hepatocytes (Mine *et al.*, 1989); however, in pituitary somatotrophs it reduces TRH-stimulated increases in intracellular calcium levels (Kitaoka *et al.*, 1991). The very high degree of structural conservation of inhibin and activin subunits strongly argues for the importance of this family of proteins in vertebrate life.

Activin Receptors and Signal Transduction

Affinity-labelling studies have shown that radio-labeled activin can bind to two proteins of 50-60 kD and 70-80 kD, respectively, which have been named activin type I and type II receptors, respectively (Hino *et al.*, 1989). Recently, specific activin type II receptor cDNAs and genes have been cloned from mouse (Mathews and Vale, 1991; Matzuk and Bradley, 1992a), human (Matzuk and Bradley, 1992b) and *Xenopus* (Kondo *et al.*, 1991), and are highly conserved. The single chain protein comprises an extracellular ligand-binding domain, a single cross membrane domain, and an intracellular domain. The intracellular domain has unique serine/threonine specific kinase activity predicted from the consensus amino acid sequence. Radioreceptor assay using COS cells transfected with the cloned mouse activin type II receptor cDNA showed that the binding of the iodinated activin A could be displaced by activin B with slightly lower potency and inhibin A with about 10-fold lower potency. TGF- β , another member of the polypeptide family, did not bind to the cloned activin type II receptor (Mathews and Vale, 1991). The displacement of labeled activin A by inhibin A is in agreement with the

previous report that inhibin, but not TGF- β , could compete for activin binding sites in some activin-responsive cell types (Kondo *et al.*, 1989). Although the receptor specific for inhibin has not been identified, receptors may exist that have a preferential binding affinity for inhibin (Woodruff *et al.*, 1992)

Regulation of Inhibin and Activin Expression and Production

With the development of specific radioimmunoassays for inhibin and nucleic acid hybridization techniques for measuring inhibin and activin subunit mRNAs, a lot of work has been done on the regulation of expression and production of inhibin and activin. In the ovary, inhibin production by granulosa cells is dependent on the stage of follicular development with the preovulatory follicles producing the largest amount of inhibin compared to follicles of other stages (Hillier *et al.*, 1991; Jih *et al.*, 1993). Extensive *in vitro* and *in vivo* studies have shown that pituitary FSH is the major stimulator of inhibin biosynthesis and secretion in both sexes in rats and humans (Bicsak *et al.*, 1986, 1987, 1988; Gonzales *et al.*, 1988; Handelsman *et al.*, 1989; Hillier *et al.*, 1991; Lee *et al.*, 1981, 1982; Rivier *et al.*, 1985; Suzuki *et al.*, 1987; Toebosch *et al.*, 1988; Tsonis *et al.*, 1988; Zhang *et al.*, 1987), and the actions of FSH are mediated mainly by cAMP (Bicsak *et al.*, 1987; Najmabadi *et al.*, 1993a, 1993b). FSH treatment of cultured rat granulosa cells dramatically increases mRNA expression of α , β A and β B subunits of inhibin and activin by 60, 70 and 66 fold, respectively (Turner *et al.*, 1989). As indicated previously, the reciprocal actions between pituitary FSH and gonadal inhibin constitute a closed-loop feedback system.

In addition to FSH, many other hormones or factors are also involved in modulating gonadal inhibin and activin production and secretion. As ovarian follicles mature, inhibin production by rat (Bicsak *et al.*, 1986; Suzuki *et al.*, 1987; Ying *et al.*,

1987; Zhang *et al.*, 1987) and monkey (Hillier *et al.*, 1989) granulosa cells becomes increasingly responsive to direct stimulation of LH and hCG, in agreement with the appearance of LH receptors on granulosa cells of mature follicles. Insulin-like growth factor-I (IGF-I) (Toebosch *et al.*, Bicsak *et al.*, 1986; Zhang *et al.*, 1987), insulin (Bicsak *et al.*, 1986; Suzuki *et al.*, 1987), TGF- β (LaPolt *et al.*, 1989; Bicsak *et al.*, 1987) and vasoactive intestinal peptide (VIP) (Bicsak *et al.*, 1986) also stimulate or potentiate FSH-stimulated inhibin production. GnRH (Bicsak *et al.*, 1986), adenosine (Conti *et al.*, 1988) and β -endorphin (Morris *et al.*, 1987) exert inhibitory effects on inhibin production. Epidermal growth factor (EGF) (Morris *et al.*, 1988) stimulates inhibin production by Sertoli cells but inhibits its production by granulosa cells. Gonadal steroids are also involved in the regulation of inhibin production, but the results of these studies are inconsistent (Bicsak *et al.*, 1988; Hillier, *et al.*, 1991; Morris *et al.*, 1988; Suzuki *et al.*, 1987). E2 treatment increased inhibin α and βA but not βB mRNA levels in cultured granulosa cells (Turner *et al.*, 1989), suggesting that, although α , βA and βB subunits are often co-regulated, mechanisms do exist for the differential regulation of these subunits. The precise action mechanisms of these modulators are not clear and remain to be further elucidated.

Activin binding sites have been demonstrated in ovarian granulosa cells (LaPolt *et al.*, 1989), suggesting autocrine roles for ovarian activin on granulosa cell functions. Activin stimulates or enhances FSH-stimulated inhibin and activin expression and production in granulosa cells (Doi *et al.*, 1992; Sugino *et al.*, 1988; LaPolt *et al.*, 1989). While treatment with activin alone has no effects on intracellular cAMP levels, activin potentiates the FSH and forskolin-stimulated cAMP production (LaPolt *et al.*, 1989). Inhibin alone has no effects on the gene expression of its own subunits (LaPolt *et al.*, 1989). The autocrine actions of activin on granulosa cells provides another example for an intrinsic activin-mediated regulatory system within an organ.

Inhibin and Activin Binding Proteins

The diverse functions and widespread production sites of inhibin and activin strongly suggest that mechanisms must exist to control the distribution of the biological activities of this family of polypeptides in different organs and tissues to avoid interference of endocrine, paracrine and autocrine actions among different tissues or organs. The co-production of specific binding proteins for inhibin and activin in various tissues where this family of proteins are produced and function may be one of the mechanisms for fine-tuning the local biological activities of this family of proteins. The differential expression of specific binding proteins for inhibin and activin in different tissues may play critical roles in regulating the activities of these regulators temporally and spatially to meet the specific needs of each organ system.

Follistatin Two years after isolation of inhibin, another novel protein named FSH-suppressing protein (FSP) or follistatin, was simultaneously purified from pFF and bFF by two laboratories (Robertson *et al.*, 1987; Ueno *et al.*, 1987). Similar to inhibin, follistatin can specifically inhibit FSH, but not LH, release and mRNA production by cultured rat pituitary cells (Carroll *et al.*, 1989; Robertson *et al.*, 1987; Ueno *et al.*, 1987; Ying *et al.*, 1987). In contrast to inhibin, follistatin is a single chain glycosylated protein with molecular weight of 32-35 kd, and has no structural relation to inhibin and activin family (Sharon and diZerga, 1989; Ying, 1988a). The gene sequences encoding for rat, human and porcine follistatin have been cloned and the deduced amino acid sequences exhibit structural homology to human pancreatic trypsin inhibitor and EGF (Esch *et al.*, 1987; Shimasaki *et al.*, 1988, 1989). Like inhibin and activin, the effects of follistatin on FSH secretion can only be observed after long term incubation in static culture of dispersed pituitary cells (Ying *et al.*, 1987). Co-treatment

of cultured pituitary cells with follistatin and inhibin exhibits an additive inhibitory effect on FSH release (Sharon and diZerga, 1989).

Besides the ovary, extragonadal follistatin gene expression has been demonstrated in rat kidney, brain (Shimasaki *et al.*, 1989), decidual tissue (Kaiser *et al.*, 1990) and, more interestingly, the pituitary as well (Gospodarowicz *et al.*, 1989). Follistatin has recently been purified from bovine pituitary cell culture and demonstrated to be produced by pituitary Stellate-follicular cells (Gospodarowicz *et al.*, 1989). Therefore, the possibility exists that follistatin may modulate FSH secretion locally within the pituitary via paracrine mechanisms.

Preoccupation with inhibin and activin has diverted attention from the importance of follistatin until recently, when a unique activin-binding protein with specific and high affinity for activin was purified from rat ovary, and surprisingly proven to be follistatin (Nakamura *et al.*, 1990). An activin-binding protein purified from bovine pituitary tissue has also been characterized to be follistatin, which specifically binds human activin A in a 1:1 molar ratio (Kogawa *et al.*, 1991). Mixing of activin and follistatin in an appropriate proportion before application to cultured pituitary cells abolishes both the stimulatory effects of activin and the inhibitory effects of follistatin on FSH secretion (Kagawa *et al.*, 1991; Krummen, *et al.*, 1993). Follistatin also neutralizes the action of activin on hemoglobin production by K-562 cells (Krummen, *et al.*, 1993). Similar to its effects on activin bioactivities, follistatin also interferes with activin immunoreactivity in some immunoassays (Krummen, *et al.*, 1993).

Since activin and follistatin are both produced in the pituitary, a hypothesis has been put forward that the observed inhibitory effects of follistatin on FSH secretion *in vitro* are probably due to its binding to the pituitary-derived activin to block the stimulatory effects of the latter (Corrigan *et al.*, 1991). Similar to the effects of specific monoclonal antibodies against activin, administration of exogenous follistatin can

suppress the post-ovariectomy hypersecretion of FSH from the allografted rat pituitary, which has been believed to be due to the increased production of activin within the pituitary (DePaolo, 1991). Therefore, the local concentration of follistatin within the pituitary could fine-tune the stimulatory actions of activin on FSH release, which may explain the occasional failure of exogenous activin to elicit FSH release *in vivo*, even though the plasma levels of activin after administration reach as high as 1050 ng/ml (Shintani *et al.*, 1991).

In situ hybridization and immunohistochemistry demonstrate that follistatin and its mRNA exist in ovarian granulosa cells and luteal cells (Nakatani *et al.*, 1991). The expression and production of ovarian follistatin fluctuate during folliculogenesis and the estrous cycle, and PMSG administration dramatically increases the expression of follistatin mRNA (Nakatani *et al.*, 1991; Shimasaki *et al.*, 1989). Immunoreactive follistatin can only be detected in granulosa cells of healthy dominant follicles, and its mRNA expression in atretic follicles dramatically decreases with no immunoreactivity for follistatin detectable in these follicles (Nakatani *et al.*, 1991). Taken together the reports that activin can induce follicle atresia (Woodruff *et al.*, 1990) and follistatin is a specific activin-binding protein that can abolish the actions of activin (Nakamura *et al.*, 1990), it is conceivable that ovarian activin and its binding protein, follistatin, may play important physiological roles in follicle selection and atresia induction. Follistatin may bind to activin within the follicles to neutralize the atresia-inducing actions of activin, therefore, preventing the follicles from atresia (Nakatani *et al.*, 1991). Thus, the relative expression and production of these two proteins may form an intrinsic local regulatory system within the follicles to determine the destination of follicular development. What remains interesting is the factors that regulate the local expression of activin and its binding protein, follistatin.

α -2 Macroglobulin The binding activities for activin and inhibin have also

been demonstrated recently in human serum with the putative binding protein migrating with α -2 class globulins in electrophoresis (Krummen *et al.*, 1993; Vaughan and Vale, 1993). This serum activin and inhibin binding protein has been characterized to be α -2 macroglobulin on the basis that [^{125} I]-activin or -inhibin binds to purified human and bovine α -2 macroglobulin (Krummen *et al.*, 1993; Vaughan and Vale, 1993), and that preadsorption with antiserum to α -2 macroglobulin decreases the binding capacity of human serum (Krummen *et al.*, 1993). In contrast to follistatin, α -2 macroglobulin does not have significant effects on either bioactivity or immunoreactivity of activin and inhibin (Krummen *et al.*, 1993). The physiological significance of α -2 macroglobulin as activin and inhibin binding protein *in vivo* remains unclear. Preliminary evidence suggests roles for α -2 macroglobulin in delivering the proteins to the target organs and reducing their clearance rate in the plasma, thus prolonging the half-lives, of activin and inhibin without affecting their biological activities (Krummen *et al.*, 1993; Vaughan and Vale, 1993).

Other Gonadal Proteins and Peptides

α -inhibin and β -inhibin

Two novel peptides with inhibin-like activity have been purified from human seminal plasma and are designated " α -inhibin" and " β -inhibin", respectively (Ramasharma *et al.*, 1984; Sheth *et al.*, 1984). These two peptides are chemically different from the dimeric inhibin of follicular fluid origin. α -inhibin is a 92 amino acid peptide which also gives rise to two other smaller peptides of 31 and 52 amino acids (Ramasharma *et al.*, 1984; Li *et al.*, 1985), and β -inhibin has 94 amino acids (Seidah *et*

et al., 1984). Furthermore, the *in vitro* bioassay used for detection of α - and β -inhibin activities is whole mouse pituitary culture rather than the commonly used dispersed cell culture (Ramasharma *et al.*, 1981; Rivier *et al.*, 1987a). Neither α -inhibin nor β -inhibin exhibits inhibin activity in the cultures of dispersed pituitary cells (Li and Ramasharma, 1987; Vale *et al.*, 1988). Injection of the natural and synthetic α -inhibin suppresses the postcastration rise of serum FSH levels (Yamashiro *et al.*, 1984). The mechanisms whereby α - and β -inhibin inhibit FSH secretion remain unclear. The binding sites for α -inhibin have been identified in human pituitary membrane preparations (Li and Ramasharma, 1987) and appear to be distinct from GnRH binding sites (Sairam *et al.*, 1981); however, β -inhibin has been reported to be capable of displacing GnRH from its binding sites on pituitary cell membranes (Sheth *et al.*, 1982; Vijayalakshmi *et al.*, 1981). Administration of β -inhibin to male rats reduces GnRH receptor number in the pituitary (Sheth *et al.*, 1982). Very interestingly, although β -inhibin itself has inhibitory actions on pituitary FSH release, a synthetic nonapeptide (Thr-Cys-Ser-Val-Ser-Glu-Trp-Gly-Ile) representing the amino acid sequence 86-94 of β -inhibin preferentially enhances the basal release of FSH and potentiates GnRH-stimulated FSH and LH release from rat pituitaries (Hurkadli *et al.*, 1989). This nonapeptide has been successfully used to induce ovulation in a teleostean species, the rice-field eel, *Monopterus albus* (Homeshwor *et al.*, 1991).

Gonadotropin Surge-Inhibiting Factor (GnSIF)

As mentioned above, inhibin activity has been demonstrated in follicular fluids from several species. Suppression of basal FSH release from cultured rat pituitary cells by FFs has been commonly used as the *in vitro* inhibin bioassay. Recently, a novel protein factor distinct from inhibin has been identified in the ovary. It does not affect

basal secretion of GTHs (Sopelak and Hodge, 1984; Schenken and Hodgne, 1986) but inhibits GnRH-stimulated FSH and LH secretion both *in vivo* and *in vitro*, and the surge release of FSH and LH caused by estrogen positive feedback (Littman and Hodgen, 1984; Schenken *et al.*, 1984; Danforth *et al.*, 1987). This novel protein, now called gonadotropin surge-inhibiting factor (GnSIF), from pFF can be separated from inhibin in chromatography (Danforth *et al.*, 1987). The biochemical characteristics and physiological significance of GnSIF remain to be further elucidated.

Others

Besides activin, other gonadotropin-stimulating peptide factors have been identified in mammalian gonads. Ying *et al.* (1981) purified a 3.5 kD peptide from rat follicular fluid with gonadotropin-releasing activity. In bull seminal plasma, three non-activin proteins, BSP-A1, A2 and A3 of molecular weight 20 kD, were purified and were shown to have stimulatory effects on both FSH and LH secretion *in vitro* (Manjunath, 1984). A FSH-stimulating peptide has been purified from human follicular fluid and designated human follicular gonadotropin-releasing peptide (hF-GRP). The amino acid sequence of this novel peptide (Thr-Asp-Thr-Ser-His-His-Asp-Gln-Asp-His-Pro-Thr-Phe-Asn) is different from that of hypothalamic GnRH (Li *et al.*, 1987). hF-GRP is active in both whole rat pituitary incubation and dispersed pituitary cell culture.

From these lines of work, it is evident that mammalian gonads produce not only inhibin and activin, but also many other protein or peptide factors that are implicated in the regulation of pituitary GTH release. Since these factors are all different from each other, it is possible that they may be involved in the regulation of different aspects of GTH secretion during the reproductive cycle.

Recent Developments in Non-mammalian Vertebrates

To date studies on the gonadal proteins or peptides that modulate pituitary GTH secretion have been largely limited to mammals. By using a mammalian bioassay system, Tsonis *et al.* (1988) demonstrated, for the first time, the existence of inhibin-like activity in a nonmammalian vertebrate, the domestic hen. As in the mammalian ovary, chicken inhibin is also produced by granulosa cells (Tsonis *et al.*, 1988; Vanmontfort *et al.*, 1992). The plasma concentrations of immunoreactive inhibin in the domestic hen dramatically decrease when the ovary becomes atrophied, suggesting that the ovary is the major source of serum inhibin (Vanmontfort *et al.*, 1992). GTHs, especially LH, are stimulatory to inhibin production in the hen (Johnson *et al.*, 1993; Vanmontfort *et al.*, 1992).

As mentioned above, the most exciting development in lower vertebrates regarding inhibin and activin family has been the recent demonstration of existence of activin in *Xenopus* and its capability of inducing mesoderm formation during early embryonic development (Albano *et al.*, 1990; Asashima *et al.*, 1991; Smith *et al.*, 1990; Thomsen *et al.*, 1990; van den Eijnden-Van Raaij *et al.*, 1990). These studies show that activin subunits are highly conserved compared to those of mammalian species. Unfortunately, although *Xenopus* activin is capable of stimulating rat FSH release *in vitro* (Smith *et al.*, 1990), nothing is known about its actions on *Xenopus* GTH release.

There is no information available on the existence and functions of inhibin and activin family of hormones in teleosts. A few investigators have attempted to demonstrate the presence of an inhibin-like regulatory mechanism in teleosts; however,

progress has been limited. By using perfusion of *Tilapia* pituitary fragments, Rubin-Kedem *et al.* (1989) failed to demonstrate any inhibitory activity in *Tilapia* seminal plasma and testicular extract; however, they found two non-steroidal factors stimulating *Tilapia* GTH secretion (Rubin-Kedem *et al.*, 1989). Detailed studies about these factors are not available, and no further characterization of these factors has been reported.

Questions Addressed in the Present Research

During the past few years, the inhibin and activin family of hormones and their involvement in the regulation of reproduction and other physiological and developmental processes have become one of the most exciting research areas in endocrinology and developmental biology. As reviewed above, inhibin and activin have been established as important components in the regulation of reproduction in mammals via endocrine, paracrine and autocrine pathways. Although significant differences exist between mammalian and teleostean reproductive regulatory systems, they do share considerable similarities regarding neuroendocrine control and gonadal feedback, both of which have been extensively studied in these two groups of vertebrates. Unfortunately, no information is available in teleosts, the largest group of vertebrates, regarding the existence and involvement of gonadal proteins such as inhibin and activin in the regulation of reproduction. On the other hand, since inhibin and activin include a wide variety of biological effects, some of which are fundamental to the life of the animal, it would be interesting to know how this family of proteins evolved structurally and functionally during vertebrate evolution. As initial work on this topic in the fishes, I propose to address the following two basic questions in the present study using goldfish

as a model: (1) do goldfish gonads produce non-steroidal substances that can regulate GTH release? and specifically (2) is inhibin and activin family of proteins present in goldfish and involved in the regulation of GTH release? (Fig. 1.2)

In this general introduction, I have provided an overall review on inhibin and activin family of hormones and other gonadal proteins and peptides in mammalian species, and the present research status in non-mammalian vertebrates. In the following chapters, the questions raised above will be addressed from different perspectives using a variety of approaches. Chapter Two provides lines of evidence for the existence of non-steroidal substances in goldfish gonadal aqueous extracts that may be involved in the regulation of pituitary GTH release. Chapter Three presents a series of experiments showing the effects of a mammalian gonadal preparation, pFF, and purified porcine inhibin and activin on GTH release in goldfish. The putative goldfish inhibin and activin subunits and their localization in gonads were visualized immunocytochemically using domain-specific antibodies against mammalian molecules, and these results are presented in Chapter Four. Chapter Five describes the molecular cloning and sequencing of goldfish activin and inhibin subunits, and, therefore, provides the direct evidence for the existence of inhibin and activin family of hormones and their molecular structures in the goldfish. Although feedback regulation of pituitary GTH secretion by gonadal non-steroidal substances has been the main focus of the present research, results presented in Chapter Six point to another potential mode of actions for goldfish activin and inhibin family of hormone, i.e., paracrine and autocrine actions within the pituitary on GTH and GH secretion. Finally, the results obtained in the present study and their possible physiological significances are summarized and discussed in Chapter Seven.

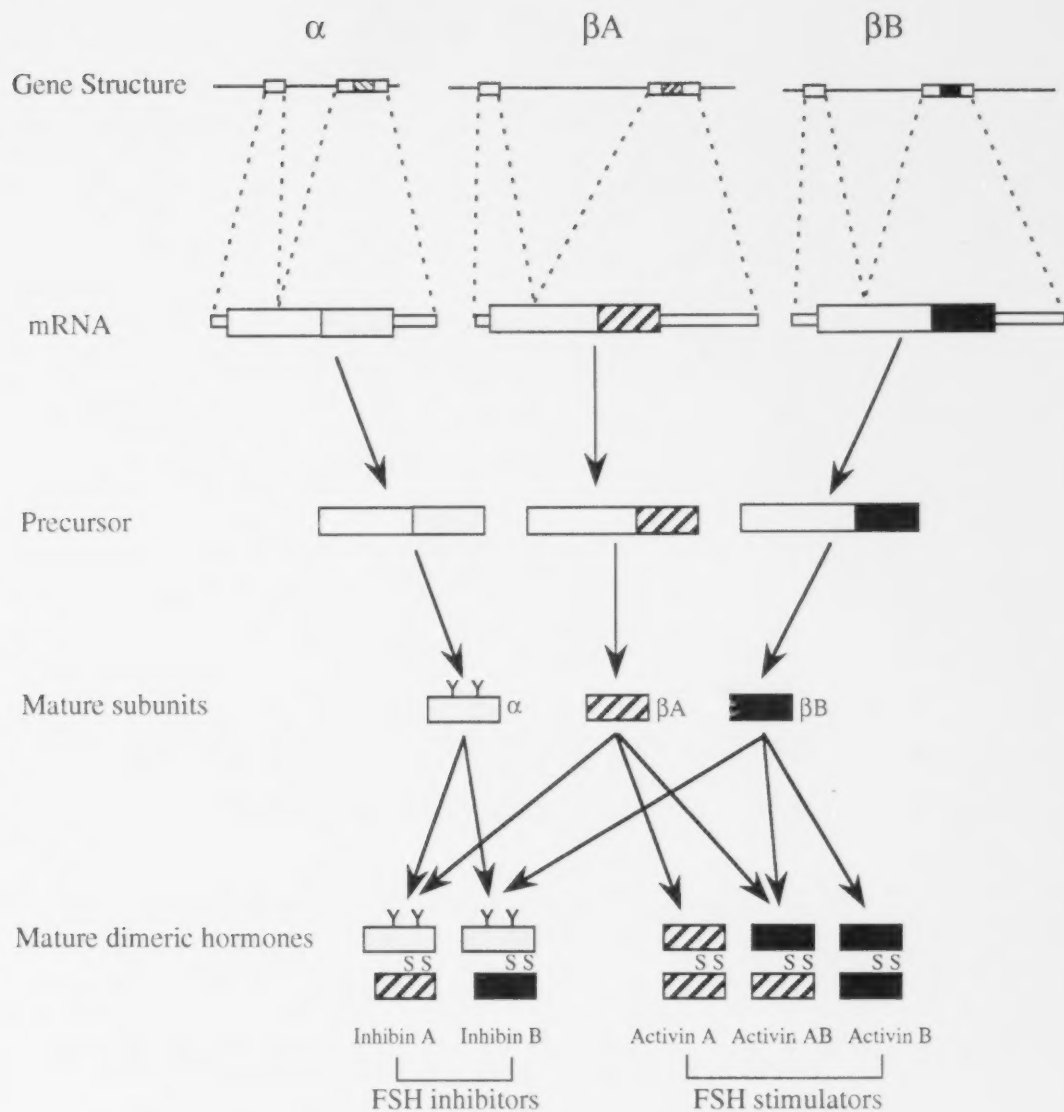


Fig. 1.1. Genomic and molecular structures of inhibin and activin. The genomic structures of rat and human α , βA and βB subunits are according to Feng *et al.* (1989) and Mason *et al.* (1987, 1989). Y, carbohydrate moieties.

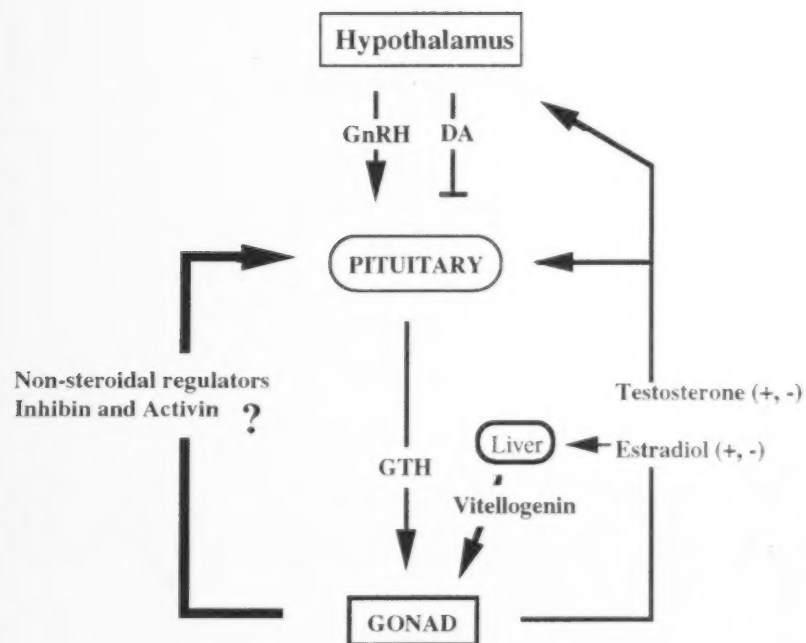


Fig. 1.2. Diagrammatic representation of the reproductive regulatory system in the goldfish and the questions addressed in the present study

References

- Abeyawardene, S.A., Vale, W.W., Marshall, G.R. and Plant, T.M. 1989 Circulating inhibin α concentrations in infant, prepubertal, and adult male rhesus monkeys (*Macaca mulatta*) and in juvenile males during premature initiation of puberty with pulsatile gonadotropin-releasing hormone treatment. *Endocrinology* 125:250-256
- Albano, R.M., Godsave, S.F., Huylebroeck, D., van Nimmen, K., Isaacs, H.V., Slack, J.M.W. and Smith, J.C. 1990 A mesoderm-inducing factor produced by WEHI-3 murine myelomonocytic leukemia cells is activin-A. *Development* 110:435-443
- Asashima, M., Nakano, H., Uchiyama, H., Sugino, H., Nakamura, T., Eto, Y., Ejima, D., Nishimatsu, S-I., Ueno, N. and Kinoshita, K. 1991a Presence of activin (erythroid differentiation factor) in unfertilized eggs and blastulae of *Xenopus laevis*. *Proc Natl Acad Sci USA* 88:6511-6514
- Asashima, M., Uchiyama, H., Nakano, H., Eto, Y., Djima, D., Sugino, H., Davids, M., Plessow, S., Born, J., Hoppe, P., Tiedemann, H. and Tiedemann, H. 1991b The vegetalizing factor from chicken embryos: its EDF (activin A)-like activity. *Mechanisms of Development* 34:135-141
- Attardi, B., Keeping, H.S., Winters, S.J., Kotsuji, F., Maurer, R.A. and Troen, P. 1989a Rapid and profound suppression of messenger ribonucleic acid encoding follicle-stimulating hormone β by inhibin from primate Sertoli cells. *Mol Endocrinol* 3:280-287
- Attardi, B., Keeping, H.S., Winters, S.J., Kotsuji, F. and Troen, P. 1989b Effect of inhibin from primate Sertoli cells and GnRH on gonadotropin subunit mRNA in rat pituitary cell cultures. *Mol Endocrinol* 3:1236-1242
- Attardi, B., Vaughan, J. and Vale, W. 1992 Regulation of FSH β messenger ribonucleic acid levels in the rat by endogenous inhibin. *Endocrinology* 130:557-559
- Attardi, B. and Winter, S.J. 1993 Decay of follicle-stimulating hormone- β messenger RNA in the presence of transcriptional inhibitors and/or inhibin, activin, or follistatin. *Mol Endocrinol* 7:668-680
- Au, C.L., Robertson, D.M. and de Kretser, D.M. 1985 Effects of hypophysectomy and subsequent FSH and testosterone treatment on inhibin production by adult rat testes. *J Endocrinol* 105:1-6
- Bardin, C.W., Morris, P.L., Chen, C.L., Shaha, C., Voglmayr, J., Rivier, J., Spiess, J. and Vale, W. 1987 Testicular inhibin: structure and regulation by FSH, androgens and EGF. In: *Inhibin-Non-steroidal Regulation of Follicle Stimulating*

Hormone Secretion. (Burger, H.G., de Kretser, D.M., Findlay, J.K., Igarashi, M., eds.). Raven Press, New York. 42:179-190

- Bhasin, S., Krummen, K.A., Swerdloff, R.S., Morelos, B.S., Kim, W.H., DiZerega, G.S., Ling, N., Esch, F., Shimasaki, S. and Toppari, J. 1989 Stage dependent expression of inhibin α and β B subunits during the cycle of the rat seminiferous epithelium. *Endocrinology* 124:987-991
- Bicsak, T.A., Tucker, E.M., Cappel, S., Vaughan, J., Rivier, J., Vale, W. and Hsueh, A.J.W. 1986 Hormonal regulation of granulosa cell inhibin. *Endocrinology* 119:2711-2719
- Bicsak, T.A., Vale, W., Vaughan, J., Tucker, E.M., Cappel, S. and Hsueh, A.J.W. 1987 Hormonal regulation of inhibin production by cultured Sertoli cells. *Mol Cell Endocrinol* 49:211-217
- Bicsak, T.A., Cajander, S.B., Vale, W. and Hsueh, A.J.W. 1988 Inhibin: studies of stored and secreted forms by biosynthetic labeling and immunodetection in cultured rat granulosa cells *Endocrinology* 122:741-748
- Bilezikjian, L.M., Corrican, A.Z. and Vale, W. 1990 Activin-A modulates growth hormone secretion from cultures of rat anterior pituitary cells. *Endocrinology* 126:2369-2376
- Billestrup, N., Gonzales, M.C., Potter, E. and Vale, W. 1990 Inhibition of somatotroph growth and growth hormone biosynthesis by activin *in vitro*. *Mol Endocrinol* 4:356-362
- Blake, C.A. and Kelch, R.P. 1981 Administration of antiluteinizing hormone-releasing hormone serum to rats: effects on periovulatory secretion of luteinizing hormone and follicle-stimulating hormone. *Endocrinology* 109:2175-2179
- Campbell, B.K., Mann, G.E., McNeilly, A.S. and Baird, D.T. 1990 The pattern of ovarian inhibin, estradiol, and androstenedione secretion during the estrous cycle of the ewe. *Endocrinology* 127:227-235
- Campan, C.A. and Vale, W. 1988 Interaction between purified ovine inhibin and steroids on the release of gonadotropins from cultured rat pituitary cells. *Endocrinology* 123:1320-1328
- Carroll, R.S., Corrigan, A.Z., Gharib, S.D., Vale, W. and Chin, W.W. 1989 Inhibin, activin, and follistatin: regulation of follicle-stimulating hormone messenger ribonucleic acid levels. *Mol Endocrinol* 3:1969-1976
- Carroll, R.S., Corrigan, A.Z., Vale, W. and Chin, W.W. 1991 Activin stabilizes follicle-stimulating hormone-beta messenger ribonucleic acid levels. *Endocrinology* 129:1721-1726

- Cate, P.L., Mattaliano, R.J., Hession, C., Tizard, R., Farber, N.M., Cheung, A., Ninfa, E.G., Frey, A.Z., Gash, D.J., Chow, E.P., Fisher, R.A., Bertonis, J.M., Torres, G., Wallner, B.P., Ramachandran, K.L., Ragin, R.C., Manganaro, T.F., McLaughlin, D.T. and Donahow, P.K. 1986 Isolation of the bovine and human genes for Mullerian inhibiting substance and expression of the human gene in animal cells. *Cell* 45:686-694
- Chang, J.P., Van Goor, F. and Acharya, S. 1991 Influences of norepinephrine, and adrenergic agonists and antagonists on gonadotropin secretion from dispersed pituitary cells of goldfish, *Carassius auratus*. *Neuroendocrinology* 51:202-210
- Channing, C.P., Gordon, W.L., Liu, W.K. and Ward, D.N. 1985 Physiology and biochemistry of ovarian inhibin. *Proc Soc Exp Biol Med* 178:339-361
- Chappel, S.C. 1979 Cyclic fluctuation in ovarian FSH-inhibiting material in golden hamsters. *Biol Reprod* 21:447-453
- Chappel, S., Ulloa-Aguirre, A. and Coutifaris, C. 1983 Biosynthesis and secretion of follicle-stimulating hormone. *Endo Rev* 4:179-211
- Chari, S., Duraiswami, S. and Franchimont, P. 1978 Isolation and characterization of inhibin from bull seminal plasma. *Acta Endocrinol* 87:434-448
- Chari, S., Hopkinson, C.R.N., Daume, E. and Sturnz, G. 1979 Purification of inhibin from human ovarian follicular fluid. *Acta Endocrinol* 90:157-166
- Chen, C-L.C. 1993 Editorial: inhibin and activin as paracrine / autocrine factors. *Endocrinology* 132:4-5
- Conn, P.M., Huckle, W.R., Andrews, W.V. and McArdle, C.A. 1987 The molecular mechanism of action of gonadotropin releasing hormone (GnRH) in the pituitary. *Rec Prog Horm Res* 43:29-67
- Conti, M., Culler, M.D. and Negro-Vilar, A. 1988 Adenosine receptor-dependent modulation of inhibin secretion in cultured immature rat Sertoli cells. *Mol Cell Endocrinol* 59:179-186
- Corrigan, A.Z., Bilezikjian, L.M., Carroll, R.S., Bald, L.N., Schmelzer, C.H., Fendly, B.M., Mason, A.J., Chin, W.W., Schwall, R.H. and Vale, W. 1991 Evidence for an autocrine role of activin B within rat anterior pituitary cultures. *Endocrinology* 128: 1682-1684
- Crawford, R.J., Hammond, V.E., Evans, B.A., Coghlan, J.P., Haralambidis, J., Hudson, B., Penshow, J.D., Richards, R.I. and Tregear, G.W. 1987 α -inhibin gene expression occurs in the ovine adrenal cortex, and is regulated by adrenocorticotropin. *Mol Endocrinol* 1:699-706
- Cuevas, P., Ying, S-Y., Ling, N., Esch, F. and Guillemin, R. 1987 Immunohistochemical detection of inhibin in the gonad. *Biochem Biophys Res Commun* 142:23-30

- Culler, M.D. 1992 Inhibin suppresses luteinizing hormone (LH)-releasing hormone self-priming: direct action on follicle-stimulating hormone secretion and opposition of estradiol-enhanced LH secretion. *Endocrinology* 130:1605-1614
- Culler, M.D. and Negro-Vilar, A. 1988 Passive immunoneutralization of endogenous inhibin: sex-related differences in the role of inhibin during development. *Mol Cell Endocrinol* 58:263-273
- Culler, M.D. and Negro-Vilar, A. 1989 Endogenous inhibin suppresses only basal follicle-stimulating hormone secretion but suppresses all parameters of pulsatile luteinizing hormone secretion in the diestrous female rat. *Endocrinology* 124:2944-2953
- Danforth, D.R., Sinosich, M.J., Anderson, T.L., Cheng, C.Y., Bardin, C.W. and Hodgen, G.D. 1987 Identification of gonadotropin surge-inhibiting factor (GnSIF) in follicular fluid and its differentiation from inhibin. *Biol Reprod* 37:1075-1082
- de Greef, W.J., de Jong, F.H., de Koning, J., Steenbergen, J. and Van der Vaart, P.D.M. 1983 Studies on the mechanism of the selective suppression of plasma levels of follicle-stimulating hormone in the female rat after administration of steroid-free bovine follicular fluid. *J Endocrinol* 97:327-338
- de Jong, F.H. and Sharpe, R.M. 1976 Evidence for inhibin-like activity in bovine follicular fluid. *Nature* 263:71-72
- de Jong, F.H., Jansen, E.H.J.M., Hermans, W.P. and van de Molen, H.J. 1982 Purification, characterization and physiological significance of inhibin from ovarian follicular fluid. In: *Non-steroidal Regulators in Reproductive Biology and Medicine*. (Fujii, T. and Channing, C.P., eds.). Pergamon Press, Oxford. pp. 73-82
- de Jong, F.H. 1988. Inhibin. *Physiol Rev* 68:555-607
- de Kretser, D.M., McLachlan, R.I., Robertson, D.M. and Burger, H.G. 1989 Serum inhibin levels in normal men and men with testicular disorders. *J Endocrinol* 120:517-523
- Demoulin, A., Bourguignon, J.P. and Franchimont, P. 1979 "*In vitro*" inhibition of hypothalamic LH-RH by human inhibin. *Acta Endocrinol Suppl* 225:227
- DePaolo, L.V. 1991 Hypersecretion of follicle-stimulating hormone (FSH) after ovariectomy of hypophysectomized, pituitary-grafted rats: implication for local regulatory control of FSH. *Endocrinology* 128:1731-1740
- DePaolo, L.V., Bald, L.N. and Fendly, B.M. 1992 Passive immunoneutralization with a monoclonal antibody reveals a role for endogenous activin-B in mediating FSH hypersecretion during estrus and following ovariectomy of hypophysectomized, pituitary-grafted rats. *Endocrinology* 130:1741-1743
- Derynck, R., Jarrett, J.A., Chen, E.Y., Eaton, D.H., Bell, J.R., Assoian, R.K.,

- Roberts, A.B., Sporn, M.B. and Goeddel, D.V. 1985 Human transforming growth factor- β complementary DNA sequence and expression in normal and transformed cells. *Nature* 316:701-704
- de Winter, J.P., Timmerman, M.A., Vanderstichele, H.M.J., Klaij, I.A., Grootenhuys, A.J., Rommerts, F.F.G. and de Jong, F.H. 1992 Testicular Leydig cells *in vitro* secrete only inhibin α -subunits, whereas Leydig cell tumors can secrete bioactive inhibin. *Mol Cell Endocrinol* 83:105-115
- Doi, M., Igarashi, M., Hasegawa, Y., Eto, Y., Shibai, H., Miura, T. and Ibuki, Y. 1992 *In vivo* action of activin-A on pituitary-gonadal system. *Endocrinology* 130:139-144
- Eramaa, M., Heikinheimo, K., Tuuri, T., Hilden, K. and Ritvos, O. 1993 Inhibin/activin subunit mRNA expression in human granulosa-luteal cells. *Mol Cell Endocrinol* 92:R15-R20
- Erickson, G.F. and Hsueh, A.J.W. 1978 Secretion of "inhibin" by rat granulosa cells *in vitro*. *Endocrinology* 103:1960-1961
- Esch, F.S., Shimasaki, S., Cooksey, K., Mercado, M., Mason, A.J., Ying, S-Y., Ueno, N. and Ling, N. 1987 Complementary deoxyribonucleic acid (DNA) cloning and DNA sequence analysis of rat ovarian inhibins. *Mol Endocrinol* 1:388-396
- Esch, F.S., Shimasaki, S., Mercado, M., Cooksey, K., Ling, N., Ying, S., Ueno, N. and Guillemin, R. 1987 Structural characterization of follistatin: a novel follicle stimulating hormone release inhibiting polypeptide. *Mol Cell Endocrinol* 1:849-855
- Eto, Y., Tosuji, T., Takezawa, M., Takano, S., Yokogawa, Y. and Shibai, H. 1987 Purification and characterization of erythroid differentiation factor (EDF) isolated from human leukaemia cell line THP-1. *Biochem Biophys Res Commun* 142:1095-1103
- Farnworth, P.G., Robertson, D.M., de Kretser, D.M. and Burger, H.G. 1988a Effects of 31 kilodalton bovine inhibin on follicle-stimulating hormone and luteinizing hormone in rat pituitary cells *in vitro*: actions under basal conditions. *Endocrinology* 122:207-213
- Farnworth, P.G., Robertson, D.M., de Kretser, D.M. and Burger, H.G. 1988b Effects of 31 kDa bovine inhibin on FSH and LH in rat pituitary cells *in vitro*: antagonism of gonadotrophin-releasing hormone agonists. *J Endocrinol* 119:233-241
- Feng, Z.M., Li, T.P. and Chen, C.L.C. 1989 Analysis of the 5'-flanking regions of rat inhibin α and β B subunit genes suggests two different regulatory mechanisms. *Mol Endocrinol* 3:1914-1925
- Findlay, J.K., Clarke, I.J. and Robertson, D.M. 1990 Inhibin concentrations in ovarian and jugular venous plasma and the relationship of inhibin with follicle-stimulating

hormone and luteinizing hormone during the ovine estrous cycle. *Endocrinology* 126:528-535

- Forage, R.G., Ring, J.M., Brown, R.W., McInerney, B.V., Cobon, G.S., Gregson, R.P., Robertson, D.M., Morgan, F.J., Hearn, M.T.W., Findlay, J.K., Wettenhall, R.E.H., Burger, H.G. and de Kertser, D.M. 1986 Cloning and sequence analysis of cDNA species coding for the two subunits of inhibin from bovine follicular fluid. *Proc Natl Acad Sci USA* 83:3091-3095
- Franchimont, P., Croze, F., Demoulin, A., Bologne, R. and Hustin, J. 1981 Effect of inhibin on rat testicular deoxyribo-nucleic acid (DNA) synthesis *in vivo* and *in vitro*. *Acta Endocrinol* 98:312-320
- Franchimont, P., Demoulin, A. and Bourguignon, J.P. 1986 Regulation of gonadotropin secretion. In: *Male Reproductive Dysfunction, Diagnosis and Management of Hypogonadism, Infertility, and Impotence*. (Santen, R.J. and Swerdloff, R.S., eds). Marcel Dekker, New York. p. 101
- Fujii, T., Hoover, D.J. and Channing, C.P. 1983 Changes in inhibin activity, and progesterone, oestrogen and androstenedione concentrations, in rat follicular fluid throughout the oestrous cycle. *J Reprod Fertil* 69:307-314
- Gharib, S.D., Wierman, M.E., Shupnik, M.A. and Chin, W.W. 1990 Molecular biology of the pituitary gonadotropins. *Endocr Rev* 11:177-199
- Gonzales, G.F., Risbridger, G.P. and de Kertser, D.M. 1988 *In vitro* synthesis and release of inhibin in response to FSH stimulation by isolated segments of seminiferous tubules from normal adult male rats. *Mol Cell Endocrinol* 59:179-185
- Gospodarowicz, D. and Lau, K. 1989 Pituitary follicular cells secrete both vascular endothelial growth factor and follistatin. *Biochem Biophys Res Commun* 165:292-298
- Gregg, D.W., Schwall, R.H. and Nett, T.M. 1991 Regulation of gonadotropin secretion and number of gonadotropin-releasing hormone receptors by inhibin, activin-A, and estradiol. *Biol Reprod* 44:725-732
- Haisenleder, D.J., Ortolano, G.A., Dalkin, A.C., Landefeld, T.D., Vale, W.W. and Marshall, J.C. 1990 Inhibin secretion during the rat estrous cycle: relationships to FSH secretion and FSH beta subunit mRNA concentrations. *Life Sci* 47:1769-1773
- Handelsman, D.J., Spaliviero, J.A., Kidston, E. and Robertson, D.M. 1989 Highly polarized secretion of inhibin by Sertoli cells *in vitro*. *Endocrinology* 125:721-729
- Hasegawa, Y., Miyamoto, K., Iwamura, S. and Igarashi, M. 1988 Changes in serum concentrations of inhibin in cyclic pigs. *J Endocrinol* 118:211-219
- Hasegawa, Y., Miyamoto, K., Yazuki, C. and Igarashi, M. 1981 Regulation of the second surge of follicle-stimulating hormone: effects of antiluteinizing hormone-

releasing hormone serum and pentobarbital. *Endocrinology* 109:130-135

- Hedger, M.P., Drummond, A.E., Robertson, D.M., Risbridge, G.P. and de Kretser, D.M. 1989 Inhibin and activin regulate ^3H -thymidine uptake by rat thymocytes and 3T3 cells *in vitro*. *Mol Cell Endocrinol* 61:133-138
- Hillier, S.G., Wickings, E.J., Illingworth, P.I., Yong, E.L., Reichert, L.E.Jr, Baird, D.T. and McNeilly, A.S. 1991 Control of immunoactive inhibin production by human granulosa cells. *Clin Endocrinol* 35:71-78
- Hillier, S.G., Wickings, E.J., Saunders, P.T.K., Shimasaki, S., Reichert, L.E.Jr. and McNeilly, A.S. 1989 Hormonal control of inhibin production by primate granulosa cells. *J Endocrinol* 123:65-73
- Hino, M., Tojo, A., Miyazono, K., Miura, Y., Chiba, S., Eto, Y., Shibai, H. and Takaku, F. 1989 Characterization of cellular receptors for erythroid differentiation factor on murine erythroleukemia cells. *J Biol Chem* 264:10309-10314
- Hochberg, Z., Weiss, J. and Richman, R.A. 1981 Inhibin-like activity in extracts of rabbit placentae. *Placenta* 2:259-264
- Homeshwor, M., Gadre, S.V., Sheth, A.R. and Singh, H. 1991 Induced spawning of rice-field eel by a synthetic nonapeptide. In *Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish* (Scott, A.P., Sumpter, J.P., Kime, D.E. and Rolfe, M.S., eds), FishSymp, Sheffield, p. 277
- Hsueh, A.J.W., Dahl, K.D., Vaughan, J., Tucker, E., Rivier, J., Bardin, C.W. and Vale, W. 1987 Heterodimers and homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone-stimulated androgen biosynthesis. *Proc Natl Acad Sci USA* 84:5082-8086
- Hurkadli, K.S., Mahale, S.D., Iyer, K.S.N. and Sheth, A.R. 1989 Enhancement of *in vitro* release of FSH from rat pituitaries by a synthetic nonapeptide fragment of human seminal plasma inhibin. *Life Sci* 45:1357-1363
- Hutchinson, L.A., Findlay, J.R., de Vos, F.L. and Robertson, D.M. 1987 Effects of bovine inhibin, transforming growth factor β and bovine activin A on granulosa cell differentiation. *Biochem Biophys Res Commun* 146:1405-1412
- Itoh, H., Suzuki, K. and Kawauchi, H. 1988 The complete amino acid sequence of β -subunits of two distinct chum salmon GtHs. *Gen Comp Endocrinol* 71:438-451
- Jakubowiak, A., Janecki, A. and Steinberger, A. 1989 Similar effects of inhibin and cycloheximide on gonadotropin release in superfused pituitary cell cultures. *Biol Reprod* 41:454-463
- Jakubowiak, A., Janecki, A. and Steinberger, A. 1990 Action kinetics of inhibin in superfused pituitary cells depend on gonadotropin-releasing hormone treatment. *Endocrinology* 127:211-217
- Jakubowiak, A., Janecki, A., Tong, D., Sanborn, B.M. and Steinberger, A. 1991 Effects of recombinant human inhibin and testosterone on gonadotropin secretion

- and subunit mRNA in superfused male rat pituitary cell cultures stimulated with pulsatile gonadotropin-releasing hormone. *Mol Cell Endocrinol* 82:265-273
- Jih, M.H., Lu, J.K.H., Wan, Y.J.Y. and Wu, T.C. 1993 Inhibin subunit gene expression and distribution in the ovaries of immature, young adult, middle-aged, and old female rats. *Endocrinology* 132:319-326
- Johnson, P.A., Wang, S-Y. and Brooks, C. 1993 Characterization of a source and levels of plasma immunoreactive inhibin during the ovulatory cycle of the domestic hen. *Biol Reprod* 48:262-267
- Kaipia, A., Parvinen, M., Shimasaki, S., Ling, N. and Toppari, J. 1991 Stage-specific cellular regulation of inhibin α -subunit mRNA expression in the rat seminiferous epithelium. *Mol Cell Endocrinol* 82:165-173
- Kaiser, M., Gibori, G. and Mayo, K.E. 1990 The rat follistatin gene is highly expressed in decidual tissue. *Endocrinology* 126:2768-2770
- Kitaoka, M., Kojima, I. and Ogata, E. 1989 A stimulatory effect of somatostatin: enhancement of activin A-mediated FSH secretion in rat pituitary cells. *Biochem Biophys Res Commun* 162:958-962
- Kitaoka, M., Takano, K., Tanaka, Y., Kojima, I., Teramoto, A. and Ogata, E. 1991 Inhibition of growth hormone secretion by activin A in human growth hormone-secreting tumour cells. *Acta Endocrinol* 124:666-671
- Kling, O.R., Roche, P.C., Campeau, J.D. Nishimura, K., Nakamura, I.M. and diZerega, G.S. 1984 Identification of a porcine follicular fluid fraction which suppresses follicular response to gonadotropins. *Biol Reprod* 30:564-572
- Knight, P.G. and Castillo, R.J. 1988. Effects of bovine follicular fluid on gonadotrophin secretion in intact and chronically ovariectomized ewes before and after desensitization of pituitary gonadotrophs to gonadotrophin-releasing hormone. *J Endocrinol* 117:431-439
- Kobayashi, M. and Stacey, N.E. 1990 Effects of ovariectomy and steroid hormone implantation on serum gonadotropin levels in female goldfish. *Zool Sci* 7:715-721
- Koegh, E.J., Lee, V.W.K., Rennie, G.C., Burger, H.G., Hudson, B. and de Kretser, D.M. 1976 Selective suppression of FSH by testicular extracts. *Endocrinology* 98:997-1004
- Kogawa, K., Nakamura, T., Sugino, K., Takio, K., Titani, K. and Sugino, H. 1991 Activin-binding protein is present in pituitary. *Endocrinology* 128:1434-1440
- Koiter, T.R., Schaaf-Verdonk, G.C.J., Kuiper, H., Pols-Valkhof, N., Schunling, G.A. 1983 Control of follicle-stimulating hormone secretion by steroid-free bovine follicular fluid in the ovariectomized rat. *J Endocrinol* 99:1-8
- Kondo, M., Hashimoto, M., Etoh, Y., Murata, M., Shibai, H. and Muramatsu, M. 1989 Identification of the two types of specific receptor for activin/EDF expressed

on Friend leukemia and embryonal carcinoma cells. *Biochem Biophys Res Commun* 161:1267-1272

- Kondo, M., Tashiro, K., Fujii, G., Asano, M., Miyoshi, R., Yamada, R., Muramatsu, M. and Shiokawa, K. 1991 Activin receptor mRNA is expressed early in *Xenopus* embryogenesis and the level of the expression affects the body axis formation. *Biochem Biophys Res Commun* 181:684-690
- Kotsuji F., Winters, S.J., Keeping, H.S., Attardi, B., Oshima, H. and Troen, P. 1988 Effects of inhibin from primate Sertoli cells on follicle-stimulating hormone and luteinizing hormone release by perfused rat pituitary cells. *Endocrinology* 122:2796-2802
- Krummen, L.A., Woodruff, T.K., DeGuzman, G., Cox, E.T., Baly, D.L., Mann, E., Garg, S., Wong, W-L, Cossum, P. and Mather, J.P. 1993 Identification and characterization of binding proteins for inhibin and activin in human serum and follicular fluids. *Endocrinology* 132:431-443
- LaPolt, P.S., Soto, D., Su, J.G., Campen, C.A., Vaughan, J., Vale, W. and Hsueh, A.J.W. 1989 Activin stimulation of inhibin secretion and messenger RNA levels in cultured granulosa cells. *Mol Endocrinol* 3:1666-1673
- Laws, S.C., Beggs, M.J., Webster, J.C. and Miller, W.L. 1990 Inhibin increases and progesterone decreases receptors for gonadotropin-releasing hormone in ovine pituitary culture. *Endocrinology* 127:373-380
- Lee, V.W.K., McMaster, J., Quigg, H., Findlay, J. and Leversh, L. 1981 Ovarian and peripheral blood inhibin concentrations increase with gonadotrophin treatment in immature rats. *Endocrinology* 108:2403-2405
- Lee, V.W.K., McMaster, J., Quigg, H. and Leversha, L. 1982 Ovarian and circulating inhibin levels in immature female rats treated with gonadotrophin and after castration. *Endocrinology* 111:1849-1854
- Li, C.H., Hammonds, R.G. Jr., Ramasharma, K. and Chung, D. 1985 Human seminal alpha inhibins: isolation, characterization and structure. *Proc Natl Acad Sci USA* 82:4041-4044
- Li, C.H. and Ramasharma, K. 1987 Inhibin. *Ann Rev Pharmacol Toxicol* 27:1-21
- Li, C.H., Ramasharma, K., Yamashiro, D. and Chung, D. 1987 Gonadotropin-releasing peptide from human follicle fluid: isolation, characterization, and chemical synthesis. *Proc Natl Acad Sci USA* 84:959-962
- Li, S-Q, Hu, Z-Q and Ying, S-Y. 1992 GH₃ pituitary cells synthesize inhibin, activin, and activin receptor. Program and Abstracts of 74th Annual Meeting of The Endocrine Society p. 102 (abstract 202)
- Ling, N., Ying, S.Y., Ueno, N., Esch, F., Denoroy, L. and Guillemin, R. 1985 Isolation and partial characterization of a Mr 32,000 protein with inhibin activity from porcine follicular fluid. *Proc Natl Acad Sci USA* 82:7217-7221

- Ling, N., Ying, S-Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M. and Guillemin, R. 1986 Pituitary FSH is released by a heterodimer of the β subunits of the two forms of inhibin. *Nature* 321:779-782
- Lin, T., Calkins, J., Morris, P., Vale, W. and Bardin, W. 1989 Regulation of Leydig cell function in primary culture by inhibin and activin. *Endocrinology* 125:2134-2140
- Littman, B.A. and Hodgen, G.D. 1984 Human menopausal gonadotropin stimulation in monkeys: blockade of the luteinizing hormone surge by a highly transient ovarian factor. *Fertil Steril* 41:440-447
- Lumpkin, M.D., DePaolo, L.V. and de Negro-Vilar, A. 1984. Pulsatile release of follicle-stimulating hormone in ovariectomized rats is inhibited by porcine follicular fluid (inhibin). *Endocrinology* 114:201-206
- Manjunath, R. 1984. Gonadotropin release stimulatory and inhibitory proteins in bull seminal plasma. In: *Gonadal Proteins and Peptides and their Biological Significance*. (Sairam, M.R. and Atkinson, L.E., eds.). World Scientific, Singapore. pp 49-61
- Marder, M.L., Channing, C.P. and Schwartz, N.B. 1977 Suppression of serum follicle stimulating hormone in intact and acutely ovariectomized rats by porcine follicular fluid. *Endocrinology* 101:1639-1642
- Martin, G.B., Price, C.A., Thiery, J-C. and Webb, R. 1989 Interactions between inhibin, estradiol and progesterone in the control of gonadotropin secretion in the ewe. *J Reprod Fertil* 82:319-328
- Mason, A.J., Berkemeier, L.M., Schmelzer, C.H. and Schwall, R.H. 1989 Activin B: precursor sequences, genomic structure and *in vitro* activities. *Mol Endocrinol* 3:1352-1358
- Mason, A.J., Hayflick, J.S., Ling, N., Esch, F., Ueno, N., Ying, S-Y., Guillemin, R. Nail, H. and Seeburg, P.H. 1985 Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor β . *Nature* 318:659-663
- Mason, A.J., Nail, H.D. and Seeburg, P.H. 1986 Structure of two human ovarian inhibins. *Biochem Biophys Res Commun* 135:957-964
- Mason, A.J., Schwall, R., Renz, M., Rhee, L.M., Nikolics, K. and Seeburg, P.H. 1987 Human inhibin and activin: structure and recombinant expression in mammalian cells. In: *Inhibin-Non-steroidal Regulation of Follicle Stimulating Hormone Secretion*. (Burger, H.G., de Kretser, D.M., Findlay, J.K., Igarashi, M., eds.). Raven Press, New York. 42:77-88
- Mather, J.P., Attie, K.M., Woodruff, T.K., Rice, G.C. and Phillips, D.M. 1990 Activin stimulates spermatogonial proliferation in germ-cell cocultures from immature rat testis. *Endocrinology* 127:3206-3214
- Mathews, L.S. and Vale, W.W. 1991 Expression cloning of an activin receptor, a

predicted transmembrane serine kinase. *Cell* 65:973-982

- Matzuk, M.M. and Bradley, A. 1992a Structure of the mouse activin receptor type II gene. *Biochem Biophys Res Commun* 185:404-413
- Matzuk, M.M. and Bradley, A. 1992b Cloning of the human activin receptor cDNA reveals high evolutionary conservation. *Biochimica et Biophysica Acta* 1130:105-108
- Mayo, K.E., Cerelli, C.M., Spiess, J., Rivier, J., Rosenfeld, M.G., Evans, R.M. and Vale, W. 1986 Inhibin A-subunit cDNA from porcine ovary and human placenta. *Proc Natl Acad Sci USA* 83:5849-5853
- McCullagh, D.R. 1932 Dual endocrine activity of the testes. *Science* 76:19-20
- McDonald, J.K., Lumpkin, M.D., Samson, W.K. and McCann, S.M. 1985 Neuropeptide Y affects secretion of luteinizing hormone and growth hormone in ovariectomized rats. *Proc Natl Acad Sci USA* 82:561-564
- McLachlan, R.I., Healy, D.L., Robertson, D.M., Burger, H.G. and de Kretser, D.M. 1986 The human placenta: a novel source of inhibin. *Biochem Biophys Res Commun* 140:485-490
- McLachlan, R.I., Dahl, K.D., Bremner, W.J., Schwall, R., Schmelzer, C.H., Mason, A.J. and Steiner, R. 1989 Recombinant human activin-A stimulates basal FSH and GnRH-stimulated FSH and LH release in the adult male macaque, *Macaca fascicularis*. *Endocrinology* 125:2787-2789
- McNeilly, A.S., Tsonis, C.G. and Baird, D.T. 1988 Inhibin. *Hum Reprod* 3:45-49
- Medhamurthy, R., Abeyawardene, S.A., Culler, M.D., Negro-Vilar, A. and Plant, T.M. 1990 Immunoneutralization of circulating inhibin in the hypophysectomized clamped male rhesus monkey (*Macaca mulatta*) results in a selective hypersecretion of follicle-stimulating hormone. *Endocrinology* 126:2116-2124
- Mercer, J.E., Clements, J.A., Funder, J.W. and Clarke, I.J. 1987 Rapid and specific lowering of pituitary FSH β mRNA levels by inhibin. *Mol Cell Endocrinol* 53:251-254
- Merchenthaler, I., Celler, M.D., Petrusz, P. and Negro-Vilar, A. 1987 Immunocytochemical localization of inhibin in rat and human reproductive tissues. *Mol Cell Endocrinol* 54:239-243
- Meunier, H., Rivier, C., Evans, R.M. and Vale, W. 1988 Gonadal and extragonadal expression of inhibin α , β A, and β B subunits in various tissues predicts diverse functions. *Proc Natl Acad Sci USA* 85:247-251
- Miller, K.F., Wesson, J.A. and Ginther, O.J. 1979 Changes in concentrations of circulating gonadotropins following administration of equine follicular fluid to ovariectomized mares. *Biol Reprod* 21:867-872
- Miller, W.L. and Huang, E.S.R. 1985 Secretion of ovine luteinizing hormone *in vitro*:

- differential positive control by 17 β -estradiol and a preparation of porcine ovarian inhibin. *Endocrinology* 117:907-911
- Mine, T., Kojima, I. and Ogata, E. 1989 Stimulation of glucose production by activin-A in isolated rat hepatocytes. *Endocrinology* 125:586-591
- Mitrani, E., Ziv, T., Thomsen, G., Shimoni, Y., Melton, D.A. and Bril, A. 1990. Activin can induce the formation of axial structures and is expressed in the hypoblast of the chick. *Cell* 63: 495-501.
- Miyamoto, K., Hasegawa, Y., Fukuda, M., Iganashi, M., Kangaw, V. and Matsuo, H. 1985 Isolation of porcine follicular fluid inhibin of 32 kDa. *Biochem Biophys Res Commun* 129:396-403
- Morris, P.L., Vale, W. and Bardin, C.W. 1987 β -endorphin regulation of FSH-stimulated inhibin production is a component of a short loop system in testis. *Biochem Biophys Res Commun* 148:1513-1519
- Morris, P.L., Vale, W., Cappel, S. and Bardin, C.W. 1988 Inhibin production by primary Sertoli cell-enriched cultures: regulation by follicle-stimulating hormone, androgens, and epidermal growth factor. *Endocrinology* 122:717-725
- Murthy, S., Ramasharma, H.M.K. and Moudgal, N.R. 1979 Studies on purification of sheep testicular inhibin. *J Reprod Fertil Suppl* 26:61-70
- Muttukrishna, S. and Knight, P.G. 1990. Effects of crude and highly purified bovine inhibin (Mr 32000 form) on gonadotropin production by ovine pituitary cells in vitro: inhibin enhances gonadotropin-releasing hormone-induced release of LH. *J Endocrinol.* 127:149-159
- Najmabadi, N., Rosenberg, L.A., Yuan, Q.X., Bhatia, N., Albiston, A., Burger, H. and Bhasin, S. 1993a Transcriptional and posttranscriptional regulation of inhibin α -subunit gene expression in rat Sertoli cells by 8-bromo-cyclic-adenosine monophosphate. *Mol Cell Endocrinol* 89:469-476
- Najmabadi, N., Rosenberg, L.A., Yuan, Q.X., Reyaz, G. and Bhasin, S. 1993b Transcriptional regulation of inhibin β B messenger ribonucleic acid levels in TM.4 or primary rat Sertoli cells by 8-bromo-cyclic adenosine monophosphate. *Mol Endocrinol* 7:561-569
- Nakamura, T., Takio, K., Eto, Y., Shibai, H., Titani, K. and Sugino, H. 1990 Activin-binding protein from rat ovary is follistatin. *Science* 247:836-838
- Nakatani, A., Shimasaki, S., DePaolo, L.V., Erickson, G.F. and Ling, N. 1991 Cyclic changes in follistatin messenger ribonucleic acid and its protein in the rat ovary during the estrous cycle. *Endocrinology* 129:603-611
- Negro-Vilar, A., Ojeda, S.R. and McCann, S.M. 1979 Catecholaminergic modulation of luteinizing hormone-releasing hormone release by median eminence terminals in vitro. *Endocrinology* 104:1749-1757
- O, W.-S., Robertson, D.M. and de Kretser, D.M. 1989 Inhibin as an oocyte meiotic

inhibitor. *Mol Cell Endocrinol* 62:307-311

- Padgett, T.W., St-Johnston, R.D. and Gelbart, W.M. 1987 A transcript from a *Drosophila* pattern gene predicts a protein homologous to the transforming growth factor β family. *Nature* 325:81-84
- Peng, C., Chang, J.P., Yu, K.L., Wong, A.O-L., Van Goor, F., Peter, R.E. and Rivier, J.E. 1993 Neuropeptide-Y stimulates growth hormone and gonadotropin-II secretion in the goldfish pituitary: involvement of both presynaptic and pituitary cell actions. *Endocrinology* 132:1820-1829
- Peng, C., Huang, Y-P. and Peter, R.E. 1990 Neuropeptide Y stimulates growth hormone and gonadotropin release from the goldfish pituitary *in vitro*. *Neuroendocrinology* 52:28-34
- Peter, R.E., Chang, J.P., Nahorniak, C.S., Omeljaniuk, R.J., Sokolowska, M., Shih, S.H. and Billard, R. 1986 Interaction of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. *Rec Prog Horm Res* 42:513-547
- Peter, R.E., Yu, K-L., Marchant, T.A. and Rosenblum, P.M. 1990 Direct neural regulation of the teleost adenohypophysis. *J Expt Zool Suppl* 4:84-89
- Petraglia, F., Vaughan, J. and Vale, W. 1989 Inhibin and activin modulate the release of gonadotropin-releasing hormone, human chorionic gonadotropin, and progesterone from cultured human placenta cells. *Proc Natl Acad Sci USA* 86:5114-5117
- Ramasharma, K., Sairam, M.R. and Ranganathan, M.R. 1981 Effect of inhibin like factors on gonadotropin release by the mouse pituitary *in vitro*. *Acta Endocrinologica* 98:496-505
- Ramasharma, K., Sairam, M.R., Seidah, N.G., Chretien, M., Manjunath, P., Schiller, P.W., Yamashiro, D. and Li, C.H. 1984 Isolation, structure, and synthesis of a human seminal plasma peptide with inhibin-like activity. *Science* 223:1199-1202
- Risbridger, G.P., Clements, J., Robertson, D.M., Drummond, A.E., Muir, J., Burger, H.G. and de Kretser, D.M. 1989 Immuno- and bioactive inhibin and inhibin α -subunit expression in rat Leydig cell cultures. *Mol Cell Endocrinol* 66:119-122
- Risbridger, G.P., Robertson, D.M. and de Kretser, D.M. 1990 Current perspectives of inhibin biology. *Acta Endocrinologica (Copenh)* 122:673-682
- Rivier, C. and Vale, W. 1987b Inhibin: measurement and role in the immature female rat. *Endocrinology* 120:1688-1690
- Rivier, C. and Vale, W. 1989 Immunoneutralization of endogenous inhibin modifies hormone secretion and ovulation rate in the rat. *Endocrinology* 125:152-157
- Rivier, C., Cajander, C., Vaughan, J., Hsueh, A.J.W. and Vale, W. 1988 Age-dependent changes in physiological action, content, and immunostaining of

inhibin in male rats. *Endocrinology* 123:120-126

- Rivier, C., Rivier, J. and Vale, W. 1986 Inhibin-mediated feedback control of follicle stimulating hormone secretion in the female rat. *Science* 234:205-208
- Rivier, C., Schwall, R., Mason, A., Burton, L., Vaughan, J. and Vale, W. 1991 Effect of recombinant inhibin on luteinizing hormone and follicle-stimulating hormone secretion in the rat. *Endocrinology* 128:1548-1554
- Rivier, C., Vale, W. and Rivier, J. 1987a Studies of the inhibin family of hormones: a review. *Horm Res* 28:104-118
- Rivier, J., Spiess, J., McClintock, R., Vaughan, J. and Vale, W. 1985 Purification and partial characterization of inhibin from porcine follicular fluid. *Biochem Biophys Res Commun* 133:120-127
- Roberts, V., Meunier, H., Vaughan, J., Rivier, J., Rivier, C., Vale, W. and Sawchenko, P. 1989 Production and regulation of inhibin subunits in pituitary gonadotropes. *Endocrinology* 124:552-554
- Robertson, D.M., Foulds, L.M., Leversha, L., Morgan, F.J., Hearn, M.T.W., Burger, H.G., Wettenhall, R.E.H. and de Kretser, D.M. 1985 Isolation of inhibin from bovine follicular fluid. *Biochem Biophys Res Commun* 126:220-226
- Robertson, D.M., Klein, R., de Vos, F.L., McLachlan, R.I., Wettenhall, R.E.H., Hearn, M.T.W., Burger, H.G. and de Kretser, D.M. 1987 The isolation of polypeptides with FSH suppressing activity from bovine follicular fluid which are structurally different to inhibin. *Biochem Biophys Res Commun* 149:744-749
- Robertson, D.M., Hayward, S., Irby, D.C., Jacobsen, J.V., Clarke, L.J., McLachlan, R.I. and de Kretser, D.M. 1988a Radioimmunoassay of rat serum inhibin: changes after PMSG stimulation and gonadectomy. *Mol Cell Endocrinol* 58:1-8
- Robertson, D.M., Tsonis, C.G., McLachlan, R.I., Handelsman, D.J., Leask, R., Baird, D.T., McNeilly, A.S., Hayward, S., Healy, D.L., Findlay, J.K., Burger, H.G. and de Kretser, D.M. 1988b Comparison of inhibin immunological and in vitro biological activities in human serum. *J Clin Endocrinol Metab* 67:438-443
- Rosenblum, P.M. and Peter, R.E. 1989 Evidence for the involvement of endogenous opioids in the regulation of gonadotropin secretion in male goldfish. *Gen Comp Endocrinol* 73:21-27
- Rubin-Kedem, H., Levavi-Zermonsky, B. and Yaron, Z. 1989 Factors in tilapia testes affecting gonadotropin secretion from perfused pituitary gland: a preliminary study. *Gen Comp Endocrinol* 74:316-317
- Rush, M.E. 1985 Effects of LH-releasing hormone antagonist or lesions of the medial basal hypothalamus on periovulatory gonadotrophin release in female rats. *J*

- Rush, M.E. and Lipner, H. 1979 Blockade of gonadotropin-releasing hormone-induced secretion of pituitary follicle-stimulating hormone by inhibin-containing preparations. *Endocrinology* 105:187-194
- Sairam, M.R., Ranganathan, M.R. and Ramasharma, K. 1981 Binding of an inhibin-like protein from bull seminal plasma to ovine pituitary membranes. *Mol Cell Endocrinol* 22:251-264
- Sawchenko, P.E., Plotsky, P.M., Pfeiffer, S.W., Cunningham, E.T. Jr, Vaughan, J., Rivier, J. and Vale, W. 1988 Inhibin β in central neural pathways involved in the control of oxytocin secretion. *Nature* 334:615-617
- Schenken, R.S. and Hodgen, G.D. 1986 Follicle-stimulating hormone blocks estrogen-positive feedback during the early follicular phase in monkeys. *Fertil Steril* 45:556-560
- Schenken, R.S., Anderson, W.H. and Hodgen, G.D. 1984 Follicle-stimulating hormone increases ovarian vein nonsteroidal factors with gonadotropin-inhibiting activity. *Fertil Steril* 42:785-790
- Schwartz, N.B. and Channing, C.P. 1977 Evidence for ovarian "inhibin" suppression of the secondary rise in serum follicle stimulating hormone levels in proestrus rats by injection of porcine follicular fluid. *Proc Natl Acad Sci USA* 74:5721-5724
- Schwartz, N.B., Rivier, C., Rivier, J. and Vale, W. 1985 Effect of gonadotropin-releasing hormone antagonists on serum follicle-stimulating hormone and luteinizing hormone under conditions of singular follicle-stimulating hormone secretion. *Biol Reprod* 32:391-398
- Schubert, D., Kimura, H., LaCorbiere, M., Vaughan, J., Karr, D. and Fisher, W.H. 1990 Activin is a nerve cell survival molecule. *Nature* 344:868-870
- Schwall, R., Schmelzer, C.H., Matsuyama, E. and Mason, A.J. 1989 Multiple actions of recombinant activin-A *in vivo*. *Endocrinology* 125:1420-1423
- Scott, R.S. and Burger, H.G. 1981 Mechanism of action of inhibin. *Biol Reprod* 24:541-550
- Seethalakshmi, L., Steinberger, A. and Steinberger, E. 1984 Pituitary binding of ^3H -labeled Sertoli cell factor *in vitro*: a potential radioreceptor assay for inhibin. *Endocrinology* 115:1289-1294
- Seidah, N.G., Arabatti, N.J., Rochemont, J., Sheth, A.R. and Chretien, M. 1984 Complete amino acid sequence of human seminal plasma β inhibin. *FEBS Lett* 175:349-356
- Setchell, B.P. and Jacks, F. 1974 Inhibin like activity in rete testis fluid. *J Endocrinol* 62:675-676

- Setchell, B.P. and Sirinathsinghji, D.J. 1972 Anti-gonadotrophic activity in rete testis fluid, a possible "inhibin". J Endocrinol 53:1x-1xi
- Sharon, A.J. and diZerga, G.S. 1989 Intragonadal regulation of follicular maturation. Endocr Rev 10:205-229
- Sheth, A.R., Arabatti, N., Carlquist, M. and Jornvall, H. 1984 Characterization of a polypeptide from human seminal plasma with inhibin (inhibition of FSH secretion) like activity. FEBS Lett. 165:11-15
- Sheth, A.R., Joshi, L.R., Moodbidri, S.B. and Rao, S.S. 1979 Characterization of a gonadal factor involved in the control of FSH secretion. J Reprod Fertil Suppl 26:71-85
- Sheth, P.R., Dandekar, S.P., Seethalakshmi, N. and Sheth, A.R. 1982 Inhibin interaction with LHRH receptors at the pituitary level. Arch Androl 8:185-188
- Shimasaki, S., Koga, M., Esch, F., Cooksey, K., mercado, M., Koba, A., Ueno, N., Ying, S-Y., Ling, N. and Guillemin, R. 1988 Primary structure of the human follistatin precursor and its genomic organization. Proc Natl Acad Sci USA 85:4218-4222
- Shimasaki, S., Koga, M., Buscaglia, M.L., Simmons, D.L., Bicsak, T.A. and Ling, N. 1989 Follistatin gene expression in the ovary and extragonadal tissues. Mol Endocrinol 3:651-659
- Shintani, Y., Takada, Y., Yamasaki, R. and Saito, S. 1991 Radioimmunoassay for activin A/EDF, method and measurement of immunoreactive activin A/EDF levels in various biological materials. J Immunol Methods 137:267-274
- Sloley, B.D., Kah, O., Trudeau, V.L., Dulka, J.G. and Peter, R.E. 1992 Amino acid neurotransmitters and dopamine in brain and pituitary of the goldfish: involvement in the regulation of gonadotropin secretion. J Neurochem 58:2254-2262
- Smith, J.C., Price, B.M.J., van Nimmern, K. and Huylebroeck, D. 1990 Identification of a potent *Xenopus* mesoderm-inducing factor as a homologue of activin-A. Nature 345:729-731
- Somoza, G.M. and Peter, R.E. 1991 Effects of serotonin on gonadotropin and growth hormone release from *in vitro* perfused goldfish pituitary fragments. Gen Comp Endocrinol 82:103-110
- Sopelak, V.M. and Hodge, G.D. 1984 Blockade of the estrogen-induced luteinizing hormone surge in monkeys: a nonsteroidal, antigenic factor in porcine follicular fluid. Fertil Steril 41:108-113
- Sporn, M.B., Roberts, A.B., Wakefield, L.M. and Assoian, R.K. 1986 Transforming growth factor- β : biological function and chemical structure. Science 233:532-534
- Stewart, A.G., Milborrow, H.M., Ring, J.M., Crowther, C.E. and Forage, R.G. 1986 Human inhibin genes: genomic characterization and sequencing. FEBS Lett

- Sugino, H., Nakamura, T., Hasegawa, Y., Miyamoto, K., Abe, Y., Igarashi, M., Eto, Y., Shibai, H. and Titani, K. 1988 Erythroid differentiation factor can modulate follicular granulosa cell functions. *Biochem Biophys Res Commun* 153:281-288
- Summerville, J.W. and Schwartz, N.B. 1981 Suppression of serum gonadotropin levels by testosterone and porcine follicular fluid in castrate male rats. *Endocrinology* 109:1442-1447
- Suzuki, K., Kawauchi, H. and Nagahama, Y. 1988a Isolation and characterization of two distinct gonadotropins from chum salmon pituitary glands. *Gen Comp Endocrinol* 71:292-301
- Suzuki, K., Kawauchi, H. and Nagahama, Y. 1988b Isolation and characterization of subunits from two distinct salmon gonadotropins. *Gen Comp Endocrinol* 71:302-306
- Suzuki K., Nagahama, Y. and Kawauchi, H. 1988c Steroidogenic activities of two distinct salmon gonadotropins. *Gen Comp Endocrinol* 71:452-458
- Suzuki, T., Miyamoto, K., Hasegawa, Y., Abe, Y., Ui, M., Ibuki, Y. and Igarashi, M. 1987 Regulation of inhibin production by rat granulosa cells. *Mol Cell Endocrinol* 54:185-195
- Swanson, P., Suzuki, K., Kawauchi, H., and Dickhoff, W.W. 1991 Isolation and characterization of two coho salmon gonadotropins, GtH I and GtH II. *Biol Reprod* 44:29-38
- Thomsen, G., Woof, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W. and Melton, D.A. 1990 Activins are expressed early in *Xenopus* embryogenesis and can induce axial mesoderm and anterior structures. *Cell* 63:485-493
- Toebosch, A.M.W., Robertson, D.M., Trapman, J., Klaasen, P., de Paus, R.A., de Jong, F.H. and Grootegeed, J.A. 1988 Effects of FSH and IGF-I on immature rat Sertoli cells: inhibin α - and β -subunit mRNA levels and inhibin secretion. *Mol Cell Endocrinol* 55:101-105
- Torney, A.H., Robertson, D.M. and de Kretse, D.M. 1991 Characterization of inhibin and related proteins in bovine fetal testicular and ovarian extract: evidence for the presence of inhibin subunit products and FSH-suppressing protein. *J Endocrinol* 133:111-120
- Totsuka, Y., Tabuchi, M., Kojima, I., Shibai, H. and Ogata, E. 1987 A novel action of activin A: stimulation of insulin secretion from rat pancreatic islets. *Biochem Biophys Res Commun* 156:335-339
- Trudeau, V.L., Murthy, C.K., Habibi, H.R., Sloley, B.D. and Peter, R.E. 1993 Effects of sex steroid treatments on gonadotropin-releasing hormone-stimulated gonadotropin secretion from the goldfish pituitary. *Biol Reprod* 48:300-307

- Trudeau, V.L., Peter, R.E. and Sloley, B.D. 1991 Testosterone and estradiol potentiate the serum gonadotropin response to gonadotropin-releasing hormone in goldfish. *Biol Reprod* 44:951-960
- Tsonis, C.G., McNeilly, A.S. and Baird, D.T. 1988 Inhibin secretion by the sheep ovary during the luteal and follicular phases of the estrous cycle and following stimulation with FSH. *J Endocrinol* 117:283-292
- Turner, I.M., Saunders, P.T.K., Shimasaki, S. and Hilier, S.G. 1989 Regulation of inhibin subunit gene expression by FSH and estradiol in cultured rat granulosa cells. *Endocrinology* 125:2790-2792
- Ueno, N., Ling, N., Ying, S-Y., Esch, F., Shimasaki, S. and Guillemin, R. 1987 Isolation and partial characterization of follistatin: a single-chain Mr 35,000 monomeric protein that inhibits the release of follicle stimulatory hormone. *Proc Natl Acad Sci USA* 84:8282-8286
- Vale, W., Rivier, C., Hsueh, A., Campen, C., Meunier, H., Bicsak, T., Vaughan, J., Corrigan, A., Bardin, W., Sawchenko, P., Petraglia, F., Yu, J., Plotsky, P., Spiess, J. and Rivier, J. 1988 Chemical and biological characterization of the inhibin family of protein hormones. *Recent Prog. Horm Res* 44:1-34
- Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W. and Karr, D. 1986 Purification and characterization of a FSH releasing protein from porcine ovarian follicular fluid. *Nature* 321:776-779
- van de Eijnden-Van Raaij, A.J.M., van Zoelent, E.J.J., van Nimmen, K., Koster, C.H., Snoek, G.T., Durston, A.J. and Huylebroeck, D. 1990 Activin-like factor from a *Xenopus leavis* cell line responsible for mesoderm induction. *Nature* 345:732-734
- Van der Kraak, G., Suzuki, K., Peter, R.E., Itoh, H. and Kawauchi, H. 1992 Properties of common carp gonadotropin I and gonadotropin II. *Gen Comp Endocrinol* 85:217-229
- van Dissel-Emiliani, F.M.F, Grootenhuis, A.J., de Jong, F. and de Rooij, D.G. 1989 Inhibin reduces spermatogonial numbers in testes of adult mice and chinese hamsters. *Endocrinology* 125:1898-1903
- Vaughan, J.M. and Vale, W.W. 1993 α 2-macroglobulin is a binding protein of inhibin and activin. *Endocrinology* 132:2038-2050
- Vanmontfort, D., Rombauts, L., Decuypere, E. and Verhoeven, G. 1992 Source of immunoreactive inhibin in the chicken ovary. *Biol Reprod* 47:977-983
- Vijayalakshmi, S., Sheth, P.R., Dandekar, S.P., Vase, A.Y., Moodbidri, S.B. and Sheth, A.R. 1981 Biological studies with low and high molecular weight inhibin preparations. *Int J Androl* 4:691-702

- Wallace, J.M. and McNeilly, A.S. 1986 Changes in FSH and the pulsatile secretion of LH during treatment of ewes with bovine follicular fluid throughout the luteal phase of the oestrous cycle. *J Endocrinol* 111:317-327
- Wang, Q.F., Farnworth, P.G., Findlay, J.K. and Burger, H.G. 1988 Effect of purified 31 K bovine inhibin on the specific binding of gonadotropin-releasing hormone to rat anterior pituitary cells in culture. *Endocrinology* 123:2161-2166
- Wang, Q.F., Farnworth, P.G., Findlay, J.K. and Burger, H.G. 1989 Inhibitory effect of pure 31 kDa bovine inhibin on GnRH-induced up-regulation of GnRH binding sites in cultured rat anterior pituitary cells. *Endocrinology* 124:363-368
- Weeks, D.L. and Melton, D.A. 1987 A maternal mRNA localised to the vegetal hemisphere in *Xenopus* eggs codes for a growth factor related to TGF β . *Cell* 51:861-867
- Weiss, J., Harris, P.E., Halvorson, L.M., Crowley, W.F. Jr. and Jameson, J.L. 1992 Dynamic regulation of follicle-stimulating hormone- β messenger ribonucleic acid levels by activin and gonadotropin-releasing hormone in perfused rat pituitary cells. *Endocrinology* 131:1403-1408
- Welschen, R., Hermans, W.P., Dullaart, J. and de Jong, F.H. 1977 Effects of and inhibin-like factor present in bovine and porcine follicular fluid on gonadotrophin levels in ovariectomized rats. *J Reprod Fertil* 50:129-131
- Westhof, G., Westhof, K., Hasegawa, Y., Miyamoto, K. and diZerega, G.S. 1990 Inhibin secretion by individual porcine follicles perfused *in vitro*. *Fertil and Steril* 54:718-724
- Williams, A.T. and Lipner, H. 1981 Negative feedback control of gonadotropin secretion by chronically administered estradiol and porcine follicular fluid (gonadostatin) in ovariectomized rats. *Endocrinology* 109:1496-1501
- Winters, S.J. 1990 Inhibin is released together with testosterone by the human testis. *J Clin Endocrinol Metab* 70:548-550
- Wong, W.L., Lofgren, J., Garg, S., Bald, L., Woodruff, T. and Fendly, B. 1992 Specific immunoassays for measurement of activin A and activin B in biological fluids. Program and Abstracts of 74th Annual Meeting of The Endocrine Society p. 101 (abstract 200)
- Woodruff, T.K. and Mayo, K.E. 1990 Regulation of inhibin synthesis in the rat ovary. *Ann Rev Physiol* 52:807-821
- Woodruff, T.K., Borree, J., Attie, K.M., Cox, E.T., Rice, G.C. and Mather, J.P. 1992 Stage-specific binding of inhibin and activin to subpopulations of rat germ cells. *Endocrinology* 130:871-881
- Woodruff, T.K., Lyon, R.J., Hansen, S.E., Rice, G.C. and Mather, J.P. 1990 Inhibin and activin locally regulate rat ovarian folliculogenesis. *Endocrinology* 127:3196-

- Woodruff, T.K., Meunier, H., Jones, P.B.C., Hsueh, A.J.W. and Mayo, K.E. 1987 Rat inhibin: molecular cloning of α and β subunit complementary deoxyribonucleic acids and expression in the ovary. *Mol Endocrinol* 1:561-568
- Wozney, J.M., Rosen, V., Celeste, A.J., Mitsock, L.M., Whitters, M.J., Kriz, R.W., Hewick, R.M. and Wang, E.A. 1989 Novel regulators of bone formation: molecular clones and activities. *Science* 242:1528-1534
- Yamashiro, D., Li, C.H., Ramasharma, K. and Sairam, M.R. 1984 Synthesis and biological activity of human inhibin-like peptide (1-31). *Proc Natl Acad Sci USA* 81:5399-5402
- Ying, S.Y. 1988a Inhibins, activins, and follistatins: gonadal proteins modulating the secretion of follicle-stimulating hormone. *Endocr Rev* 9:267-293
- Ying, S.Y. 1988b Inhibins and activins. In: *Frontiers in Neuroendocrinology*. (Martini, L. and Ganong, W.F., eds.). Raven Press, New York. 10:167-184
- Ying, S-Y., Becker, A., Ling, N., Ueno, N. and Guillemin, R. 1986 Inhibin and beta type transforming growth factor (TGF β) have opposite modulating effects on the follicle stimulating hormone (FSH) induced aromatase activity of cultured rat granulosa cells. *Biochem Biophys Res Commun* 136:969-975
- Ying, S-Y., Becker, A., Swanson, G., Tan, P., Ling, N., Esch, F., Ueno, N., Shimasaki, S. and Guillemin, R. 1987 Follistatin specifically inhibits pituitary follicle stimulating hormone release *in vitro*. *Biochem Biophys Res Commun* 149:133-139
- Ying, S-Y., Ling, N., Bohlen, P. and Guillemin, R. 1981 Gonadocrinins: peptides in ovarian follicular fluid stimulating the secretion of pituitary gonadotropins. *Endocrinology* 108:1206-1215
- Yu, J., Shao, L., Lemas, V., Yu, A.L., Vaughan, J., Rivier, J. and Vale, W. 1987 Importance of FSH-releasing protein and inhibin in erythrodifferentiation. *Nature* 330:765-767
- Yu, K.L., Rosenblum, P.M. and Peter, R.E. 1991 *In vitro* release of gonadotropin-releasing hormone from the brain preoptic-anterior hypothalamic region and pituitary of female goldfish. *Gen Comp Endocrinol* 81:256-267
- Zhang, Z., Carson, R., Herington, A., Lee, V. and Burger, H.G. 1987 Follicle stimulating hormone and somatomedin C stimulate inhibin production by rat granulosa cells *in vitro*. *Endocrinology* 120:1633-1638
- Zhang, Z., Findlay, J.K., Carson, R.S., Herington, A.C. and Burger, H.G. 1988 Transforming growth factor β enhances basal and FSH-stimulated inhibin production by rat granulosa cells *in vitro*. *Mol Cell Endocrinol* 58:161-166

Chapter Two

PRELIMINARY EVIDENCE FOR EXISTENCE OF NON-STEROIDAL GONADAL REGULATOR(S) OF GONADOTROPIN RELEASE IN THE GOLDFISH

Introduction

McCullagh first described 60 years ago that animal gonads produced nonsteroidal substances that regulated pituitary GTH secretion. The term "inhibin" was proposed to designate a putative factor in the aqueous extracts of bull testes that could prevent the appearance of castration cells in the anterior pituitary gland (McCullagh 1932). The putative gonadal "inhibin" was later further demonstrated to be a selective non-steroidal inhibitor of FSH secretion. During the past decades, inhibin activity of suppressing basal FSH release has been demonstrated in various gonadal preparations such as follicular fluids (Chari *et al.*, 1979; de Jong and Sharpe, 1976; Fujii *et al.*, 1983; Koiter *et al.*, 1983; Lumpkin *et al.*, 1984; Miller *et al.*, 1979; Summerville and Schwartz, 1981; Williams and Lipner, 1981), ovarian extracts (Channing *et al.*, 1985; Chappel, 1979; Torney *et al.*, 1991), testicular extracts (Koegh *et al.*, 1976; Murthy *et al.*, 1979; Sheth *et al.*, 1979; Torney *et al.*, 1991), rete testis fluids (Bardin *et al.*, 1987) and seminal plasma (Chari *et al.*, 1978; Sheth *et al.*, 1984) from a variety of mammalian species including pigs, cows, sheep, rodents, horses and humans.

With the development of a specific bioassay to monitor the purification process for inhibin, four laboratories simultaneously purified inhibin molecules from porcine (Ling *et al.*, 1985; Miyamoto *et al.*, 1985; Rivier *et al.*, 1985) and bovine follicular

fluid (Robertson *et al.*, 1985) in 1985. During the separation of inhibin from porcine follicular fluid, another protein was isolated that stimulates FSH release from cultured rat pituitary cells and was named FSH releasing protein (FRP) (Vale *et al.*, 1986) or activin (Ling *et al.*, 1986). Biochemical characterization revealed that activin is structurally related to inhibin (Ling *et al.*, 1986; Vale *et al.*, 1986). The stimulatory actions of activin in the gonadal preparations are believed to be overridden and masked by the potent inhibitory actions of inhibin on FSH release, which results in an overall inhibitory effect of the aqueous extracts from gonads. In addition to inhibin and activin, a variety of other protein or peptide factors of gonadal origin which had either stimulatory or inhibitory effects on pituitary GTH release have been identified in mammals (Li *et al.*, 1987; Manjunath, 1984; Ramasharma *et al.*, 1984; Schenken and Hodgen, 1986; Sheth *et al.*, 1984; Sopelak and Hodgen, 1984; Ying *et al.*, 1981); however, studies on these factors are fragmentary compared to those on inhibin and activin.

The purpose of this study was to provide preliminary evidence for the presence of peptides or proteins in the goldfish gonads that may regulate pituitary GTH secretion. The present work serves as an initial point for future studies on the identities of such molecules. A protocol similar to that used in mammals was adopted in the present work; however, a pituitary fragment perfusion system was used as the bioassay instead of static culture of pituitary cells. In common carp, a closely related species to goldfish, two chemically distinct GTHs have been identified and designated GTH-I and GTH-II; however, unlike mammalian FSH and LH, which have distinct biological functions, carp GTH-I and GTH-II possess the same biological activities in goldfish, in term of stimulation of gonadal steroidogenesis and induction of oocyte final maturation (Van der Kraak *et al.*, 1992). Since the radioimmunoassay for GTH-I was not available, the studies presented in this chapter and the rest of this thesis were based on the measurement of GTH-II using carp GTH-II as the standard in the assay.

Materials and Methods

Experimental Animals

Common or comet variety goldfish (*Carassius auratus*) of mixed sex (approximately 25 g) were purchased from Grassyfork Fisheries Co., Martinsville, IN, USA or Ozark Fisheries, Stoutland, MO, USA. Fish were acclimated to $17^{\circ}\text{C} \pm 1$ and a simulated natural photoperiod of Edmonton, and fed a commercial trout food for at least one week before use. Animals were anaesthetized with 0.05% tricaine methanesulphonate before handling.

Tissue Extracts

Mature goldfish ovaries were homogenized with a Polytron homogenizer and extracted in phosphate-buffered saline (PBS, 50 mM + 0.8% NaCl, pH 7.4) (1:1.5, weight:volume) containing 0.01 M phenylmethylsulfonyl fluoride (PMSF, a proteinase inhibitor) for 3 hours at 4°C . The homogenates were centrifuged for 1 hour at 20,000 g. The supernatants were treated with Dextran-coated charcoal (30 mg/ml charcoal, 3 mg/ml Dextran) for 30 minutes to remove endogenous steroids. After centrifugation, the supernatants were dialysed (with 12 kD cut-off) against double distilled water for 24 hours, lyophilized, and stored at -20°C for subsequent use (Fig. 2.1). Testosterone and estradiol levels in the crude goldfish ovarian extract (CGOE) were 0.12 ± 0.05 and 0.20 ± 0.11 ng/10 mg CGOE, respectively.

Extracts from the ovaries of sexually regressed and recrudescing fish, testes and muscles were prepared following the same procedures described above. The ovarian fluid was collected by centrifuging the ovulated eggs. CGOEs prepared from regressed, recrudescing and mature ovaries are designated CGOE-reg, CGOE-rec and CGOE-mat, respectively. The ovary was judged at dissection as regressed if no vitellogenic oocytes

were visually evident, as recrudescing if vitellogenic oocytes could be seen, and as mature (=prespawning) if the ovary appeared full of large yolky oocytes.

Perifusion of pituitary fragments

For bioassay, an *in vitro* pituitary perifusion system (Fig. 2.2) was used as described by Marchant *et al* (1987). Briefly, the whole pituitaries were collected from goldfish of mixed sexes and cut into small fragments ($< 0.05 \text{ mm}^3$) with a McIlwain tissue chopper. The fragments were then washed three times with medium M199 and loaded between two layers of Cytodex beads in mini-columns (3 pituitaries/column). The fragments were perifused with medium M199 with Hank's salts, 0.35 g/litre NaHCO_3 , 25 mM Hepes, antibiotics and 0.1% BSA (M199-H-BSA, pH 7.2) at 17°C. The effects of long-term and short-term exposure to CGOE of the perifused pituitary fragments on GTH-II secretion were examined. For the long-term approach, the pituitary fragments were exposed to CGOE-mat containing medium (1 mg/ml) for 24 hours at the perifusion rate of 5 ml/hour. The perifusion rate was increased to 15 ml/hour 2 hours before collecting the media. A series of 2 minute 20 nM sGnRH pulses were applied at 60 minute intervals during the period of experiment. The treatment with CGOE-mat was terminated after the second pulse of sGnRH and the medium was changed to normal M199-H-BSA. In the short-term test, the fragments were perifused with medium M199 containing 0.1% BSA for at least 15 hours at the flow rate of 5 ml/hour before any treatments were given and the flow rate was changed to 15 ml/hour 2 hours before collecting the media. CGOEs and other tissue extracts were applied for short periods of time (2 or 20 minutes) as specified in each experiment. In both long-term and short-term tests, the same amounts of BSA were used as control. The perifusion media were collected at 5 minute intervals unless otherwise specified and the GTH-II concentrations in the fractions measured with a heterologous carp GTH II radioimmunoassay as

described by Peter *et al.* (1984) and Van der Kraak *et al.* (1992).

Data analysis

In long-term experiments, the hormone levels in the twelve fractions with the lowest values from each column were averaged and defined as the "basal" level. The hormone levels in all fractions from the same column were expressed as a percentage of this average (% basal) in order to demonstrate any long-term trends in GTH-II secretion, as well as to allow the data from different columns and different experiments to be pooled. In short-term experiments, the data were expressed as either % basal or as a percentage of average levels in the three fractions before each individual treatment was applied (% prepulse). For calculating the hormone responses, the areas under the response peaks were determined. Briefly, the average hormone concentration in the three fractions immediately before each treatment pulse (prepulse, P) was determined. Any value higher than P plus one SEM was considered to be a part of the response peak. P was subtracted from each value in the peak and the differences summed and expressed as a percentage of the basal level of the column, which was defined as the hormone response (GTH-II response).

Statistical analysis was performed by using Student's t-test or one way ANOVA followed by Fisher's least significance difference (LSD) comparison.

Results

1. Long term effects of CGOE-mat on GTH-II release

The pituitary fragments in the perfusion column were perfused with CGOE-mat (1 mg/ml) for 24 hours before the perfusates were collected (Fig. 2.3). A series of sGnRH pulses (20 nM, 2 minutes) was applied at 60 minute intervals after 24 hours

pretreatment with CGOE-mat. CGOE-mat treatment was terminated after the second sGnRH pulse. Pretreatment of goldfish pituitary fragments with CGOE-mat caused an increase in GTH-II release ranging from about 130% to 230% basal. The same amount of BSA in the control columns did not affect the basal levels of GTH-II release. Notably, after terminating perfusion with CGOE-mat and switching to normal medium M199, the basal GTH-II secretion rapidly decreased to the levels comparable to those in the control columns. In addition to the stimulatory effects on basal GTH-II secretion, continuous perfusion with CGOE-mat also potentiated sGnRH-stimulated GTH-II release; the GTH-II response to the first two sGnRH pulses in the presence of CGOE-mat was significantly greater than in the control columns. The GTH-II response decreased and became comparable to that in the control columns after the switch to normal medium M199.

2. Acute effects of CGOE-mat on GTH-II release

To demonstrate whether CGOE has any acute effects on pituitary GTH-II release, CGOE-mat of different doses was applied to goldfish pituitary fragments in perfusion in a pulse of 20 minutes (Fig. 2.4). CGOE-mat caused a rapid dose-dependent increase in GTH-II release. BSA had no effects on the release of GTH-II from the pituitary fragments.

3. Effects of other tissue extracts on GTH-II release

Similar to the ovarian preparation, the testicular extract also had strong stimulatory actions on GTH-II release while the ovarian fluid induced a relatively smaller stimulatory effect. The muscle extract caused only minor increase in GTH-II release (Fig. 2.5).

4. Seasonal changes in the effectiveness of the ovarian extracts in stimulating GTH-II release

During the reproductive cycle, the goldfish ovary undergoes major morphological and physiological changes. CGOE from different stages of ovarian development was prepared and tested in the goldfish pituitary fragment perfusion to determine if the *in vitro* stimulatory effects on GTH-II release change with the reproductive cycle. The stimulatory effects of CGOE (5 mg/ml, 2 minutes) on GTH-II release showed evident seasonality (Fig. 2.6 and 2.7). Among the three preparations, CGOE-rec was the most effective in stimulating GTH-II release compared to CGOE-reg and CGOE-mat. Interestingly, the pituitary fragments appeared to become refractory to the treatment with CGOE-rec, as the GTH-II response to the second pulse of CGOE-rec significantly decreased compared to that to the first pulse.

5. The desensitizing effect of CGOE-rec on GTH-II response

The desensitizing effect of CGOE-rec on GTH-II response observed in the above experiment was further confirmed in a separate experiment in which the GTH-II response to CGOE-rec reached a much higher level (Fig. 2.8). Two sGnRH pulses (100 nM, 2 minutes) were given as pre- and post-treatment control. The GTH-II response to the first pulse of CGOE-rec was significantly higher than that to the maximal dose of sGnRH; however, the GTH-II response to the second pulse of CGOE-rec was reduced in magnitude to a level similar to the response to sGnRH. Interestingly, the GTH-II response to the post-treatment sGnRH pulse was not affected by the action of CGOE-rec (Fig. 2.8). The desensitization of the GTH-II response to CGOE-rec was then examined with intervals of 30, 60 and 120 minutes between the two CGOE-rec pulses (Fig. 2.9). The desensitizing effect of CGOE-rec continued to occur with a pulse interval of 120 minutes. The GTH-II responses to the post-treatment sGnRH pulse remained

unchanged.

6. Seasonality of GTH-II responsiveness to CGOE-rec

Two separate experiments were performed using the pituitaries from sexually regressed (gonad wt/total body wt X 100%, gonadosomatic index, GSI= $0.90 \pm 0.93\%$; mean $\pm$ SD) and recrudescing (GSI= $3.73 \pm 3.59\%$) goldfish to determine if the seasonal gonadal cycle influences the pituitary GTH-II responsiveness to CGOE-rec (Fig. 2.10). The GTH-II responses to both sGnRH and CGOE-rec by pituitary fragments from sexually regressed goldfish were low, and the GTH-II release stimulated by CGOE-rec was similar to that induced by sGnRH. With the pituitaries from sexually recrudescing goldfish, both sGnRH and CGOE-rec caused larger GTH-II responses compared to the pituitaries from regressed fish, with the magnitude of CGOE-rec-induced GTH-II release becoming significantly higher than that of sGnRH-stimulated GTH-II response.

7. Interaction between dopamine and CGOE-rec on GTH-II release

DA is a potent hypothalamic inhibitor of basal and sGnRH-stimulated GTH-II release in goldfish (Peter *et al.*, 1986). To show whether DA affects CGOE-rec action, the perfused pituitary fragments were exposed to continuous treatment with 1 μ M DA during which the first pulse of CGOE-rec was applied (Fig. 2.11). Treatment with DA caused a slight decrease in basal GTH-II release; however, the GTH-II response stimulated by CGOE-rec was not reduced compared to that in the absence of DA (the GTH-II release by the pituitary fragments from the regressed goldfish in Fig. 2.10).

Discussion

Crude aqueous extracts from goldfish ovaries exhibit stimulatory effects on both

spontaneous as well as sGnRH-stimulated GTH-II release after long term treatment. Furthermore, the extracts from both goldfish ovaries and testes have acute stimulatory actions on pituitary GTH-II release in perfusion, with pulsatile treatment for as short as 2 minutes eliciting an immediate response of GTH-II release. This is in contrast to mammalian studies on gonadal fluids and extracts that generally have inhibitory effects on pituitary GTH, especially FSH, release, which has been attributed to the actions of inhibin. Another feature of the present results that is distinct from those in mammalian species is the acute response to CGOE and testicular extract. Although the acute effects of goldfish gonadal extracts appear to be similar to that induced by GnRH, some evidence indicates that the novel stimulatory factor(s) in goldfish gonadal extracts are different from GnRH. First, the extracts had been subjected to dialysis with 12 kD cut-off point to remove most of the small molecules including small peptides; therefore, the putative stimulatory factors in the goldfish gonadal extracts are likely to have molecular weight more than 12 kD. Second, even though CGOE-rec exhibits strong desensitizing effect on the GTH-II response to the subsequent treatment, the desensitization of the response to CGOE-rec does not reduce the GTH-II response to sGnRH stimulation, suggesting that the desensitization is specifically related to the actions of CGOE-rec and is independent of sGnRH.

One interesting aspect of the GTH-II-releasing activity of goldfish ovarian extracts is that the stimulatory actions change significantly with the developmental stages of the ovaries, with CGOE-rec being the most effective compared to CGOE-reg and CGOE-mat. Furthermore, the pituitary responsiveness to CGOE-rec also varies significantly with the development of gonads. The GTH-II responsiveness to CGOE-rec increases significantly when the fish enter sexual recrudescence and maturation, similar to the seasonal variations in responsiveness to GnRH in goldfish (Murthy and Peter, unpublished results); however, the increase in the CGOE-rec-induced GTH-II response

is even higher. The increase in the action of CGOE-rec compared to CGOE-reg and CGOE-mat, and the significantly higher GTH-II responsiveness to CGOE-rec by the pituitaries from sexually recrudescing fish correlate well with the onset of ovarian recrudescence and the associated increase in plasma GTH-II levels (Kobayashi *et al.*, 1988; Peter, 1981; Trudeau *et al.*, 1991), implying that the stimulatory factor(s) in CGOE-rec might be involved in initiating these changes.

Interestingly, although DA is potent in suppressing basal GTH-II release and inhibits GnRH-induced GTH-II secretion in goldfish (Peter *et al.*, 1986). DA does not affect CGOE-rec-stimulated GTH-II release. This suggests that there may be a stimulatory pathway for GTH-II in goldfish that is independent of the tonic inhibition by the hypothalamic DA.

In mammals, a variety of factors have been identified in gonadal preparations that can either stimulate or inhibit pituitary GTH release (Li *et al.*, 1987; Manjunath 1984; Ramasharma *et al.*, 1984; Schenkena and Hodgen, 1986; Sheth *et al.*, 1984; Sopelak and Hodgen, 1984; Ying *et al.*, 1981, Ying, 1988). Therefore, any observed actions of a crude preparation only reflect a net effect depending on the relative amounts, potencies and the complex interactions of all active substances present in the mixture. This also applies to the goldfish gonadal extracts used in the present study. With overwhelming evidence in mind for the inhibitory effects of mammalian gonadal preparations on pituitary GTH secretion, the stimulatory actions of the goldfish gonadal extracts on pituitary GTH-II release described above are unexpected, although it has been reported that *Tilapia* seminal plasma and testicular extract are also stimulatory to GTH release from the pituitary fragments in perfusion (Rubin-Kedem *et al.*, 1989). The nature of the putative stimulatory factors in goldfish gonads remains unknown and additional studies are needed to identify these factors and characterize their biochemical and biological properties.

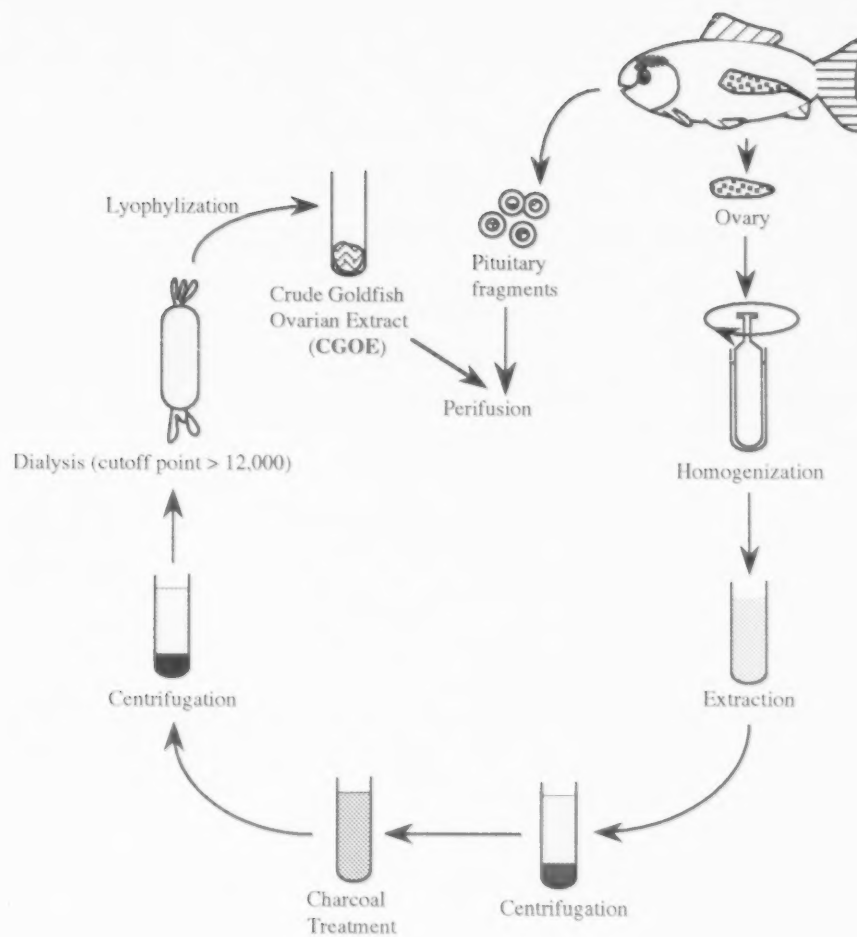


Fig. 2.1. Schematic representation of crude goldfish ovarian extract (CGOE) preparation

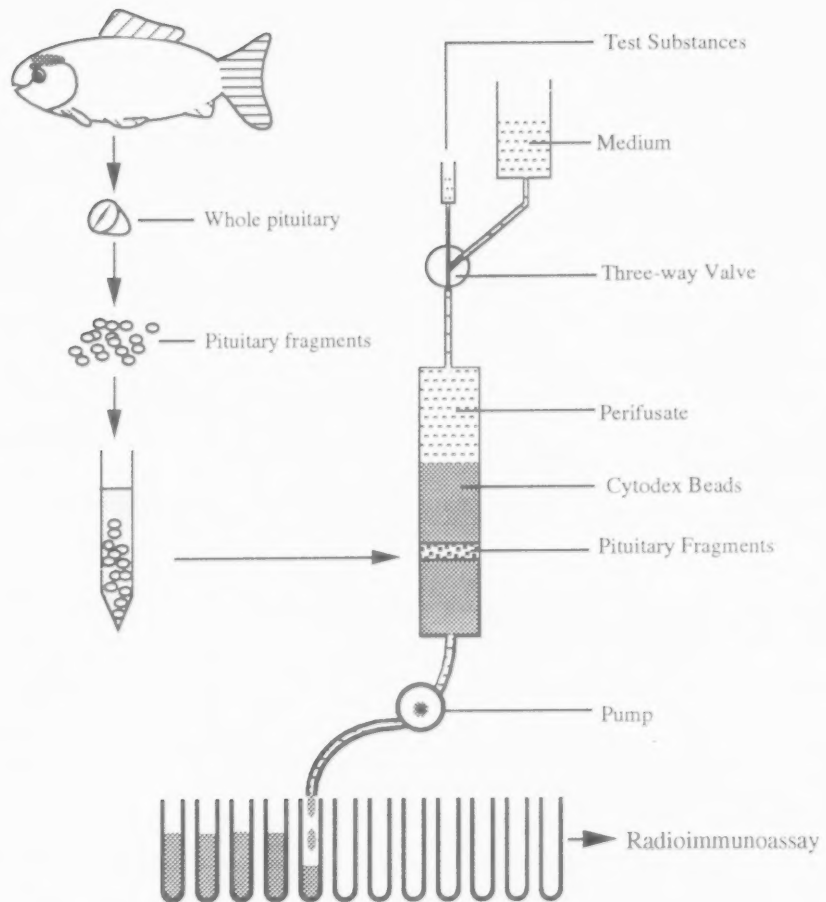


Fig. 2.2. Schematic representation of the pituitary fragment perfusion system.

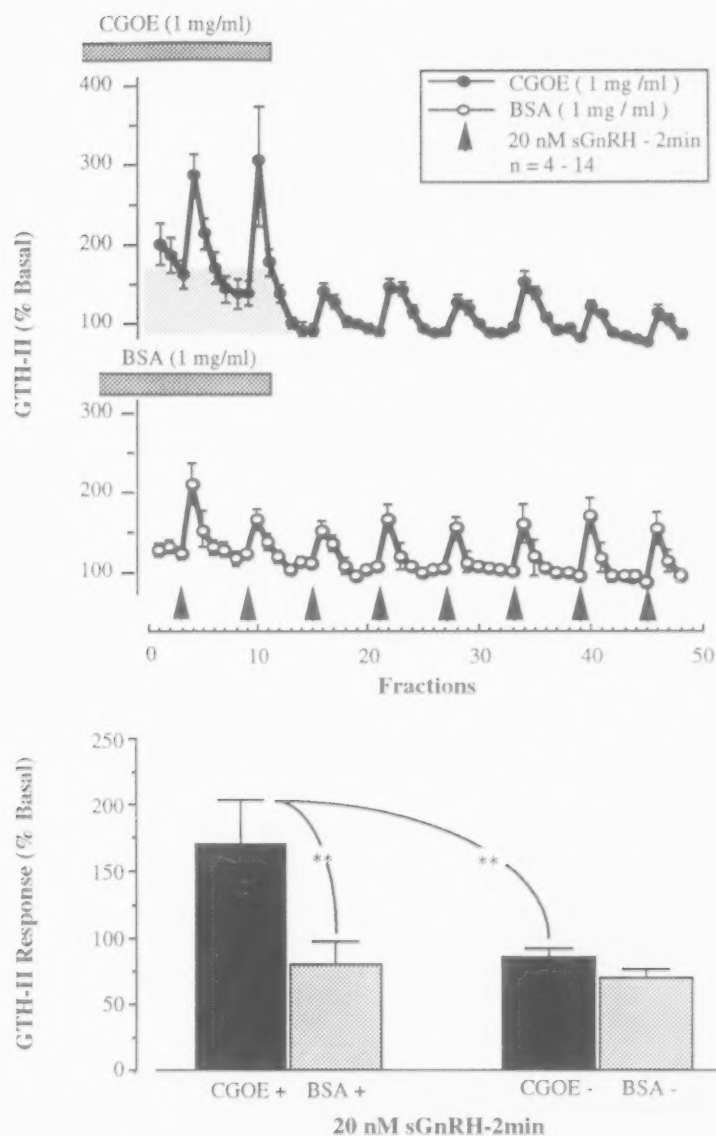


Fig. 2.3. Long-term effects of CGOE-mat on basal and sGnRH-stimulated goldfish GTH-II release by perfused pituitary fragments from sexually regressed fish. The fragments were perfused with either 1 mg/ml BSA (control) or 1 mg/ml CGOE-mat for 24 hours before collecting the media. A series of 2 minute pulses of 10 nM sGnRH was given at 60 minute intervals during the experimental period; after the second pulse of sGnRH, perfusion with CGOE-mat and BSA was discontinued, and the media changed to normal medium M199. The perfusate was collected at 10 minute intervals. Values are the average of 4-16 columns of separate experiments. Lower panel: GTH-II responses (mean $\pm$ SEM, $n=12-56$) to sGnRH quantified as described in the Materials and Methods. **Significant difference ($p<0.001$, Student's t-test).

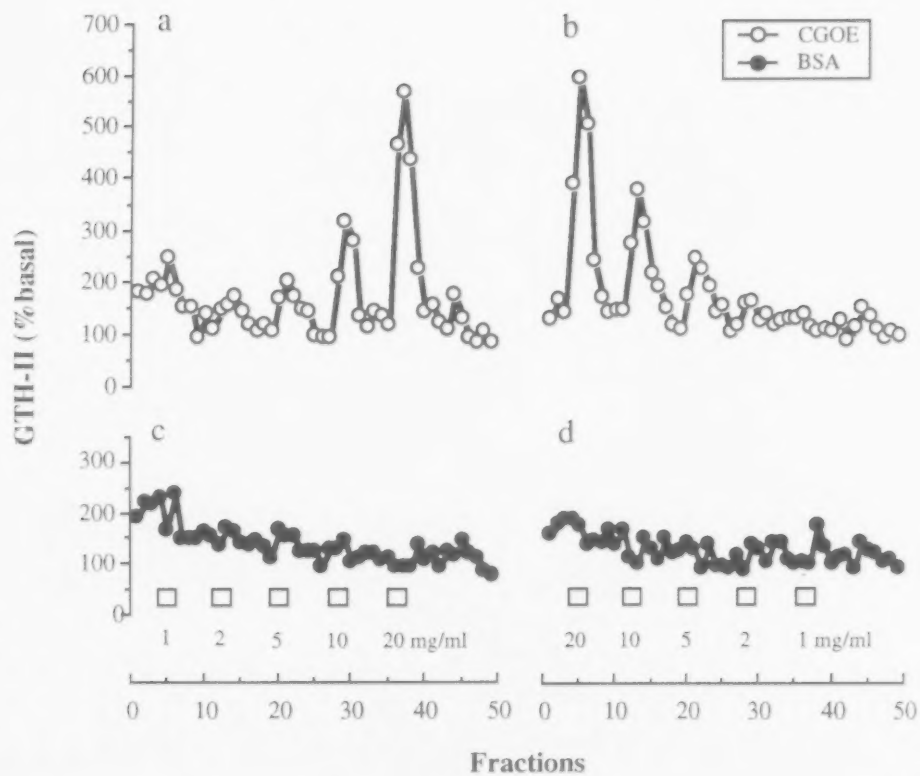


Fig. 2.4. Short-term effects of CGOE-mat on goldfish GTH-II release. CGOE-mat (a and b) and BSA (control, c and d) were applied for 20 minutes from low dose to high dose (a and c) and from high dose to low dose (c and d) in separate columns, respectively. The perfusate was collected at 10 minute intervals.

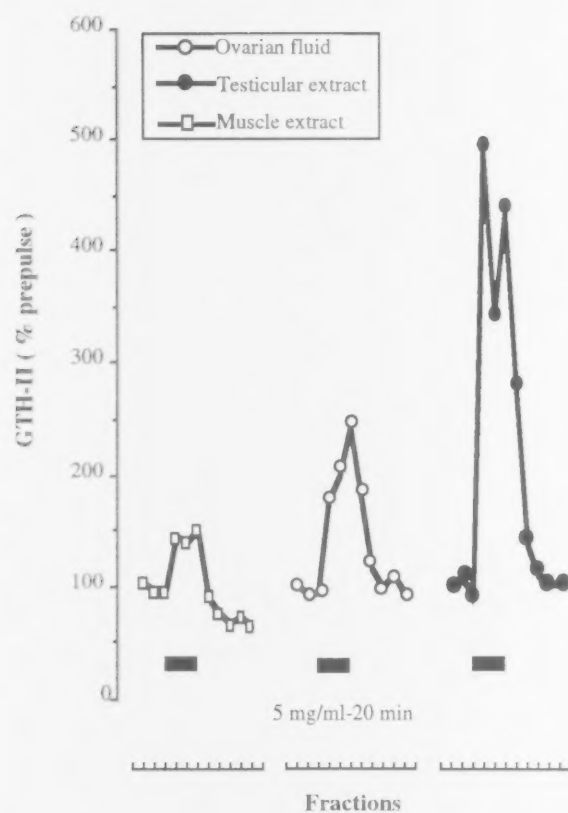


Fig. 2.5. Effects of extracts from different tissues on goldfish gonadotropin secretion. The perfusate was collected at 10 minute intervals. The values are the average of two columns.

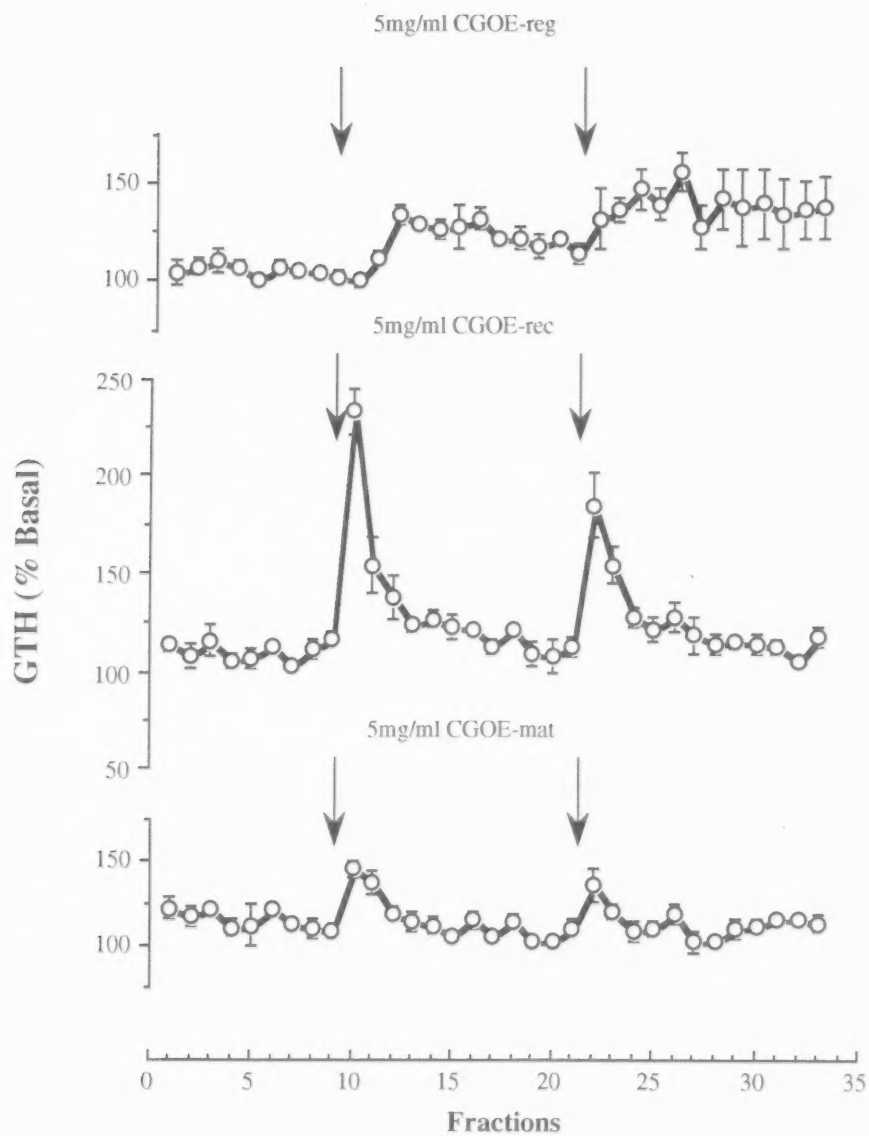


Fig. 2.6. Effects of CGOE from different ovarian stages on goldfish gonadotropin release. The values are the mean $\pm$ SEM of four columns. The pituitary were collected from early recrudescing goldfish ($GSI=1.72\pm0.51\%$).

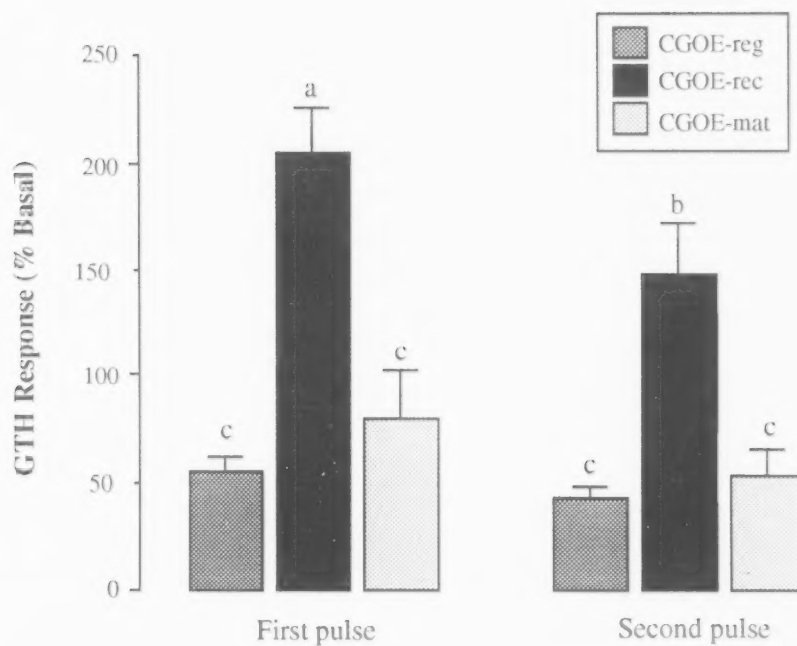


Fig.2.7. GTH-II responses (mean $\pm$ SEM, n=4) to CGOEs (5 mg/ml) from different ovarian stages quantified as described in the Materials and Methods. Different letters above each column indicate significant difference ($p < 0.05$, ANOVA followed by LSD comparison).

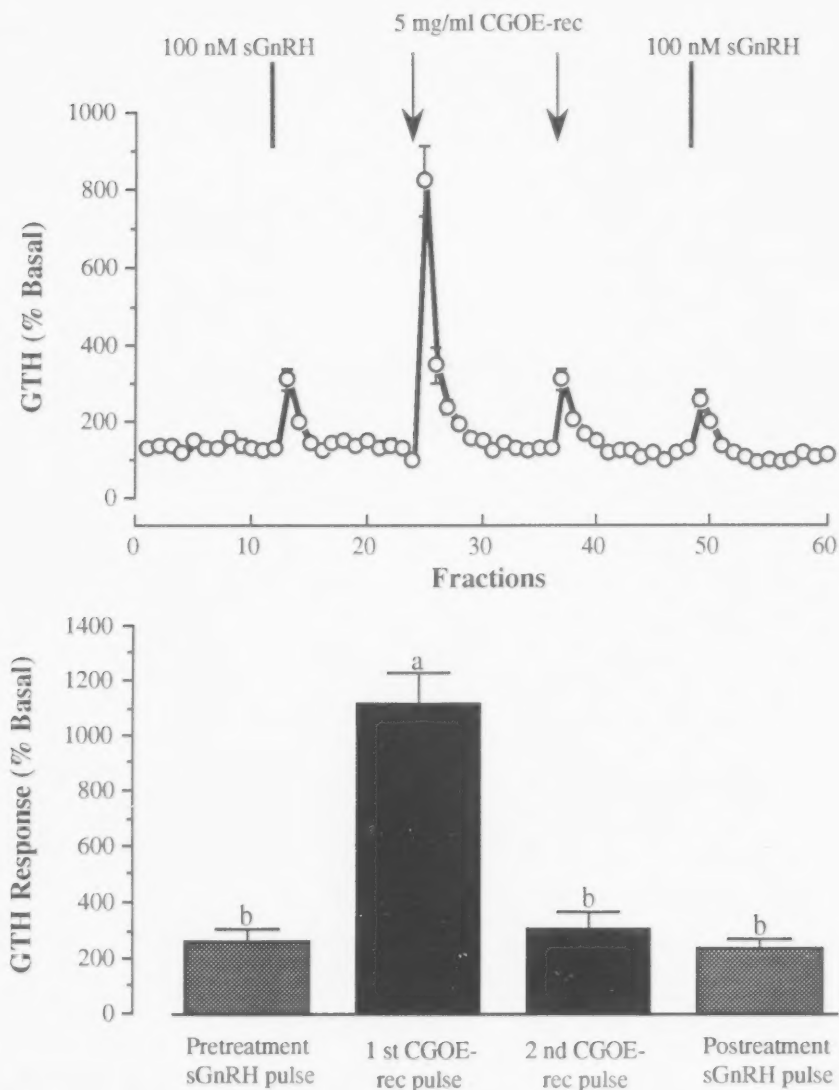


Fig. 2.8. Effects of CGOE-rec on goldfish GTH-II release by perfused pituitary fragments. After overnight preincubation, two 2 min pulses of CGOE-rec were applied to the pituitary fragments at a 60 minute interval. sGnRH (2 min pulse) was used as pre- and post-treatment control. The perfusate was collected at 5 minute intervals. Values are the average of five columns. Lower panel: GTH responses (mean $\pm$ SEM, $n=5$) quantified as described in the Materials and Methods. Different letters above each column indicate significant difference ($p<0.05$, ANOVA followed by LSD comparison).

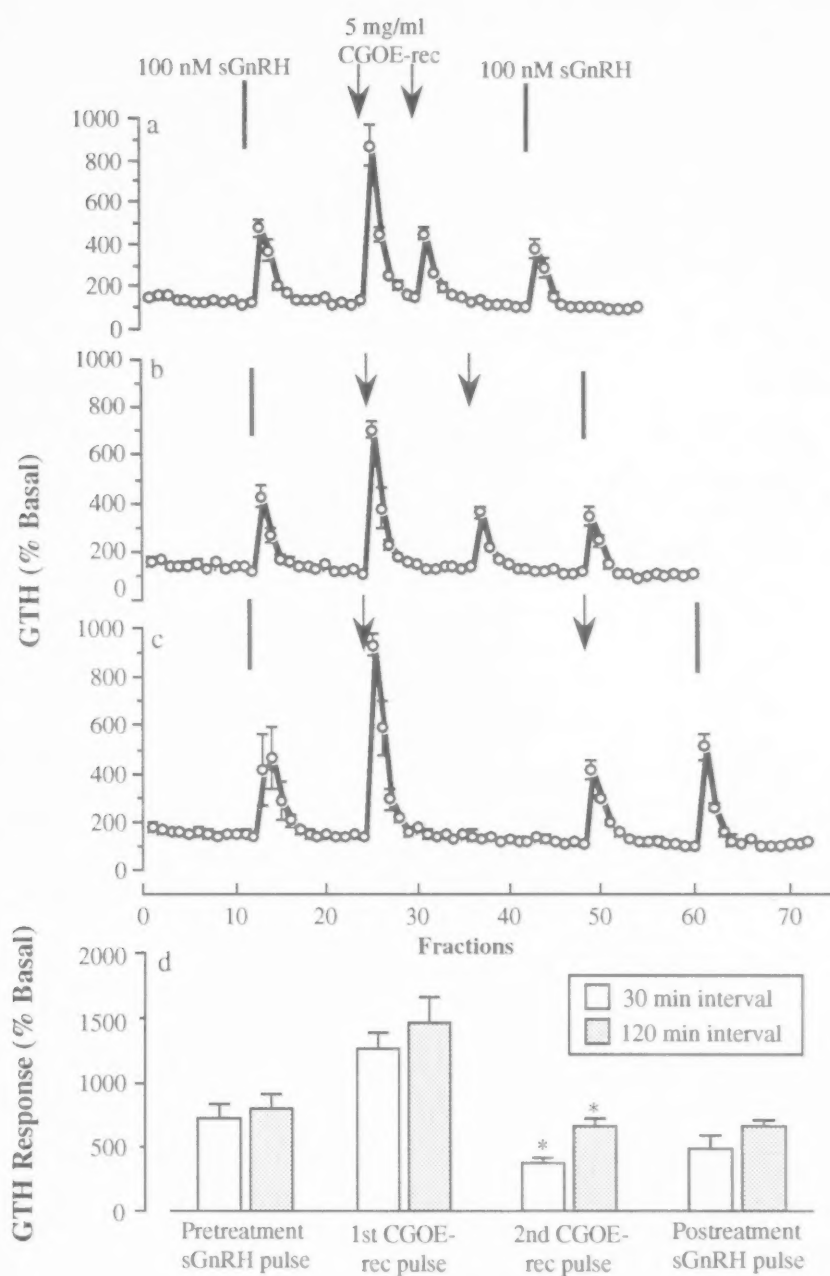


Fig. 2.9. Effects of CGOE-rec on goldfish GTH-II release by perfused pituitary fragments. Two 2 min pulses of CGOE-rec were applied to the pituitary fragments at 30 (a), 60 (b) and 120 (c) min intervals, respectively. sGnRH (2 min pulse) was used as internal pre- and post-treatment control. Values are the average of two (60 min interval) or three columns. Panel c: GTH-II responses (mean $\pm$ SEM, $n=3$) to sGnRH and CGOE-rec quantified as described in the Materials and Methods. * Significantly different from the corresponding response to the first CGOE-rec pulse ($p<0.05$, Student's t -test). The quantification for the 60 min interval was not shown because only two columns were included in this experiment.

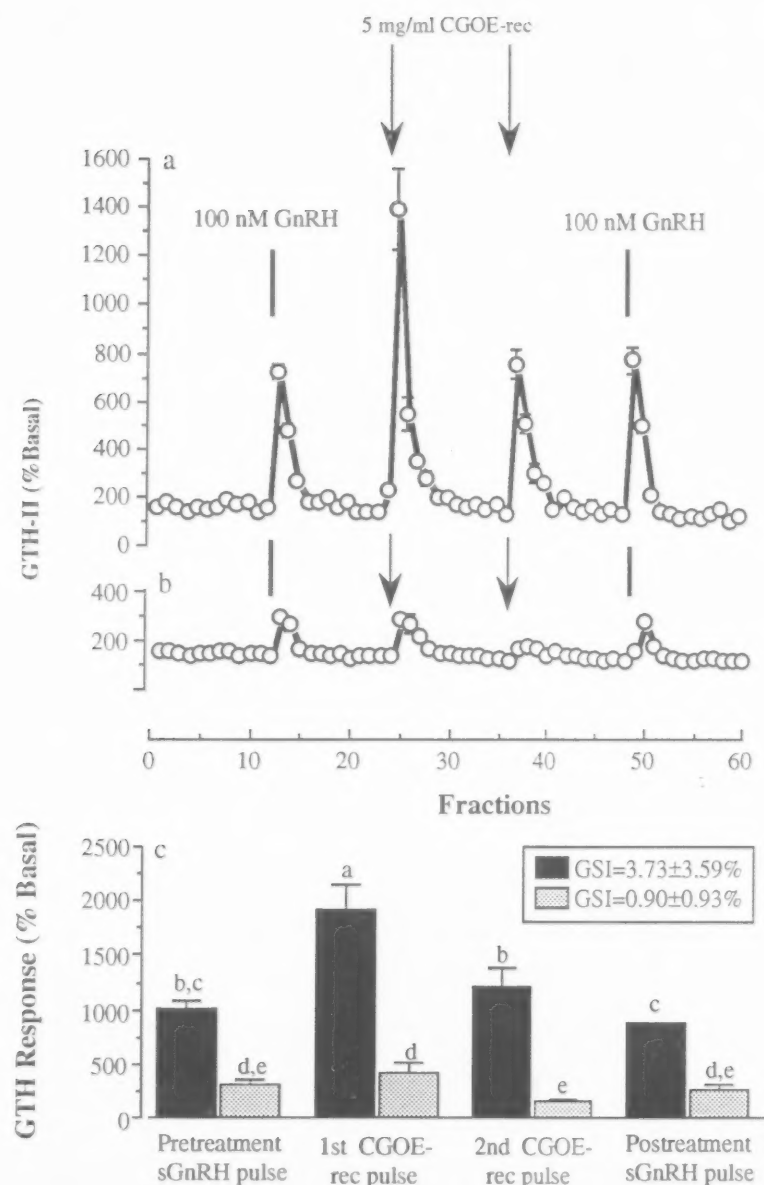


Fig. 2.10. Effects of gonad developmental stage on the pituitary GTH-II responsiveness to CGOE-rec. Two separate experiments were performed using goldfish at sexually recrudescing (a) and regressed (b) stages. Values are the average of three (a) or four (b) columns. Panel c: GTH-II responses (mean±SEM, n=3-4) to sGnRH and CGOE-rec quantified as described in the Materials and Methods. Different letters above each column indicate significant difference ($p < 0.05$, ANOVA followed by LSD comparison).

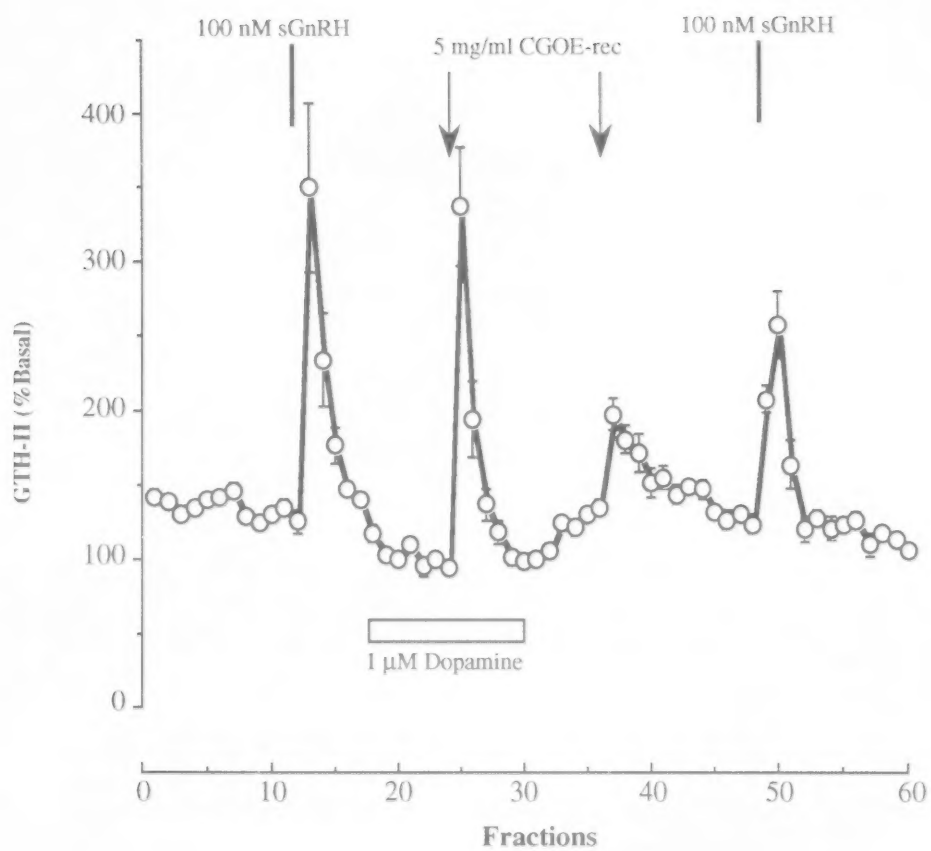


Fig. 2.11. Effects of dopamine on CGOE-rec-stimulated goldfish GTH-II release. The perfusate was collected at 5 minute intervals. Values are the average of four columns.

References

- Bardin, C.W., Morris, P.L., Chen, C.L., Shaha, C., Voglmayr, J., Rivier, J., Spiess, J. and Vale, W. 1987 Testicular inhibin: structure and regulation by FSH, androgens and EGF. In: *Inhibin-Non-steroidal Regulation of Follicle Stimulating Hormone Secretion*. (Burger, H.G., de Kretser, D.M., Findlay, J.K., Igarashi, M., eds.). Raven Press, New York. 42:179-190
- Channing, C.P., Gordon, W.L., Liu, W.K. and Ward, D.N. 1985 Physiology and biochemistry of ovarian inhibin. *Proc Soc Exp Biol Med* 178:339-361
- Chappel, S.C. 1977 Cyclic fluctuations in ovarian FSH-inhibiting material in golden hamster. *Biol Reprod* 21:447-453
- Chari, S., Duraiswami, S. and Franchimont, P. 1978 Isolation and characterization of inhibin from bull seminal plasma. *Acta Endocrinol* 87:434-448
- Chari, S., Hopkinson, C.R.N., Daume, E. and Sturnz, G. 1979 Purification of inhibin from human ovarian follicular fluid. *Acta Endocrinol* 90:157-166
- de Jong, F.H. and Sharpe, R.M. 1976 Evidence for inhibin-like activity in bovine follicular fluid. *Nature* 263:71-72
- Fujii, T., Hoover, D.J. and Channing, C.P. 1983 Changes in inhibin activity, and progesterone, oestrogen and androstenedione concentrations, in rat follicular fluid throughout the oestrous cycle. *J Reprod Fertil* 69:307-314
- Habibi, H.R. 1991 Homologous desensitization of gonadotropin-releasing hormone (GnRH) receptors in the goldfish pituitary: effects of native GnRH peptides and a synthetic GnRH antagonist. *Biol Reprod* 44:275-283
- Kobayashi, M., Aida, K. and Hanyu, I. 1988 Hormone changes during the ovulatory cycle in goldfish. *Gen Comp Endocrinol* 69:301-307
- Koegh, E.J., Lee, V.W.K., Rennie, G.C., Burger, H.G., Hudson, B. and de Kretser, D.M. 1976 Selective suppression of FSH by testicular extracts. *Endocrinology* 98:997-1004
- Koiter, T.R., Schaaf-Verdonk, G.C.J., Kuiper, H., Pols-Valkhof, N., Schunling, G.A. 1983 Control of follicle-stimulating hormone secretion by steroid-free bovine follicular fluid in the ovariectomized rat. *J Endocrinol* 99:1-8
- Li, C.H., Ramasharma, K., Yamashiro, D. and Chung, D. 1987 Gonadotropin-releasing peptide from human follicle fluid: isolation, characterization, and chemical synthesis. *Proc Natl Acad Sci USA* 84:959-962
- Ling, N., Ying, S.Y., Ueno, N., Esch, F., Denoroy, L. and Guillemin, R. 1985 Isolation and partial characterization of a Mr 32,000 protein with inhibin activity from porcine follicular fluid. *Proc Natl Acad Sci USA* 82:7217-7221
- Ling, N., Ying, S-Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M. and Guillemin, R.

- 1986 Pituitary FSH is released by a heterodimer of the β subunits of the two forms of inhibin. *Nature* 321:779-782
- Lumpkin, M.D., DePaolo, L.V. and de Negro-Vilar, A. 1984. Pulsatile release of follicle-stimulating hormone in ovariectomized rats is inhibited by porcine follicular fluid (inhibin). *Endocrinology* 114:201-206
- Manjunath, R. 1984. Gonadotropin release stimulatory and inhibitory proteins in bull seminal plasma. In: *Gonadal Proteins and Peptides and their Biological Significance*. (Sairam, M.R. and Atkinson, L.E., eds.). World Scientific, Singapore. pp 49-61
- Marchant, T.A., Fraser, R.A., Andrews, P.C. and Peter, R.E. 1987 The influence of mammalian and teleost somatostatins on the secretion of growth hormone from goldfish (*Carassius auratus* L) pituitary fragments *in vitro*. *Regul Peptides* 17:41-52
- McCullagh, D.R. 1932 Dual endocrine activity of the testes. *Science* 76:19-20
- Miller, K.F., Wesson, J.A. and Ginther, O.J. 1979 Changes in concentrations of circulating gonadotropins following administration of equine follicular fluid to ovariectomized mares. *Biol Reprod* 21:867-872
- Miyamoto, K., Hasegawa, Y., Fukuda, M., Iganashi, M., Kangaw, V. and Matsuo, H. 1985 Isolation of porcine follicular fluid inhibin of 32 kDa. *Biochem Biophys Res Commun* 129:396-403
- Murthy, S., Ramasharma, H.M.K. and Moudgal, N.R. 1979 Studies on purification of sheep testicular inhibin. *J Reprod Fertil Suppl* 26:61-70
- Peter, R.E. 1981 Gonadotropin secretion during reproductive cycles in teleosts: influences of environmental factors. *Gen Comp Endocrinol* 45:294-305
- Peter, R.E., Nahorniak, C.S., Chang, J.P. and Crim, L.W. 1984 Gonadotropin release from the pars distalis of goldfish, *Carassius auratus*, transplanted beside the brain or into the brain ventricles: additional evidence for gonadotropin-release-inhibitory factor. *Gen Comp Endocrinol* 55:337-346
- Peter, R.E., Chang, J.P., Nahorniak, C.S., Omeljaniuk, R.J., Sokolowska, M., Shih, S.H. and Billard, R. 1986 Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. *Recent Prog Horm Res* 42:513-548
- Ramasharma, K., Sairam, M.R., Seidah, N.G., Chretien, M., Manjunath, P., Schiller, P.W., Yamashiro, D. and Li, C.H. 1984 Isolation, structure, and synthesis of a human seminal plasma peptide with inhibin-like activity. *Science* 223:1199-1202
- Rivier, J., Spiess, J., McClintock, R., Vaughan, J. and Vale, W. 1985 Purification and partial characterization of inhibin from porcine follicular fluid. *Biochem Biophys Res Commun* 133:120-127
- Robertson, D.M., Foulds, L.M., Leversha, L., Morgan, F.J., Hearn, M.T.W., Burger,

- H.G., Wettenhall, R.E.H. and de Kertser, D.M. 1985. Isolation of inhibin from bovine follicular fluid. *Biochem Biophys Res Commun* 126:220-226
- Rubin-Kedem, H., Levavi-Zermonsky, B. and Yaron, Z. 1989 Factors in tilapia testes affecting gonadotropin secretion from perfused pituitary gland: a preliminary study. *Gen Comp Endocrinol* 74:316-317
- Schenken, R.S. and Hodgen, G.D. 1986 Follicle-stimulating hormone blocks estrogen-positive feedback during the early follicular phase in monkeys. *Fertil Steril* 45:556-560
- Sheth, A.R., Arabatti, N., Carlquist, M. and Jornvall, H. 1984 Characterization of a polypeptide from human seminal plasma with inhibin (inhibition of FSH secretion) like activity. *FEBS Lett.* 165:11-15
- Sheth, A.R., Joshi, L.R., Moodbidri, S.B. and Rao, S.S. 1979 Characterization of a gonadal factor involved in the control of FSH secretion. *J Reprod Fertil Suppl* 26:71-85
- Sopelak, V.M. and Hodgen, G.D. 1984 Blockade of the estrogen-induced luteinizing hormone surge in monkeys: a nonsteroidal, antigenic factor in porcine follicular fluid. *Fertil Steril* 41:108-113
- Summerville, J.W. and Schwartz, N.B. 1981 Suppression of serum gonadotropin levels by testosterone and porcine follicular fluid in castrate male rats. *Endocrinology* 109:1442-1447
- Torney, A.H., Robertson, D.M. and de Kretse, D.M. 1991 Characterization of inhibin and related proteins in bovine fetal testicular and ovarian extract: evidence for the presence of inhibin subunit products and FSH-suppressing protein. *J Endocrinol* 133:111-120
- Trudeau, V.L., Peter, R.E. and Soley, B.D. 1991 Testosterone and estradiol potentiate the serum gonadotropin response to gonadotropin-releasing hormone in goldfish. *Biol Reprod* 44:951-960
- Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W. and Karr, D. 1986 Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. *Nature* 321:776-779
- Van der Kraak, G., Suzuki, K., Peter, R.E., Itoh, H. and Kawauchi, H. 1992 Properties of common carp gonadotropin I and gonadotropin II. *Gen Comp Endocrinol* 85:217-229
- Williams, A.T. and Lipner, H. 1981 Negative feedback control of gonadotropin secretion by chronically administered estradiol and porcine follicular fluid (gonadostatin) in ovariectomized rats. *Endocrinology* 109:1496-1501
- Ying, S.Y. 1988 Inhibins, activins, and follistatins: gonadal proteins modulating the secretion of follicle-stimulating hormone. *Endocr Rev* 9:267-293
- Ying, S-Y., Ling, N., Bohlen, P. and Guillemin, R. 1981 Gonadocrinins: peptides in

ovarian follicular fluid stimulating the secretion of pituitary gonadotropins.
Endocrinology 108:1206-1215

general inhibitors such as hypothalamic GABA (Kah *et al.*, 1992) and gonadal steroids (Trudeau *et al.*, 1991, 1993).

GTH-II stimulators studied in goldfish can be grouped into three categories based on their mechanisms to counteract DA tonic inhibition, leading to increased GTH-II release. The first group includes those that have direct stimulatory actions on pituitary gonadotrophs; however, their actions are suppressed by DA. Either increased release of these stimulators or increased responsiveness of pituitary gonadotrophs helps to override the inhibitory actions of DA, thus causing increased GTH-II release. GnRH is a representative of this category (Peter *et al.* 1986). The second group is those that do not exert direct actions on pituitary gonadotrophs, but they may suppress the activities of dopaminergic neurons, therefore reducing the tonic inhibition by DA. GABA, in part, falls into this category (Trudeau *et al.*, 1993). This pathway of stimulating GTH-II release by reducing DA inhibitory tone has been demonstrated under physiological conditions by Dulka *et al.* (1992). Female pheromone 17 α ,20 β -dihydroxy-4-pregnen-3-one (17,20 β -P) stimulates a rapid increase in serum GTH-II levels in the male, and this stimulation is at least partially mediated through reduction of DA turnover in the pituitary (Dulka *et al.*, 1992). The third group of GTH-II stimulators can directly act on pituitary gonadotrophs and their actions are independent of DA inhibition. Inhibin and activin appear to belong to this category. These three modes of stimulating GTH-II release in goldfish are depicted in Fig.7.1.

In addition to hypothalamic DA and the various GTH-II stimulators, including neuropeptides such as NPY (Peng *et al.*, 1990) and neurotransmitters such as norepinephrine (Chang *et al.*, 1991), another major group of regulators involved in GTH-II secretion is the gonadal steroids. Both negative and positive aspects of steroid actions on GTH-II release have been reported in goldfish. Gonadectomy in goldfish causes a significant increase in the plasma GTH-II levels; the post-gonadectomy elevation

of GTH-II concentrations can be suppressed by estradiol or testosterone implantation (Kobayashi and Stacey, 1990), suggesting a negative role for gonadal steroids in maintaining an appropriate basal GTH level during the ovarian cycle. On the other hand, Trudeau *et al.* (1991, 1993) demonstrated that implantation of estradiol and testosterone in intact goldfish does not change basal GTH levels; however, such implantation significantly increases the GTH-II response to exogenous GnRH challenges. An abrupt release of GnRH occurs at ovulation and presumably stimulates the GTH-II surge (Yu *et al.*, 1989); the positive feedback actions of steroids on the pituitary may potentiate this surge response.

Based on our findings in the present research, I speculate that goldfish ovarian non-steroidal regulators, including inhibin and activin, may represent a new positive feedback mechanism that contributes to the increased GTH-II release that occurs during ovarian recrudescence. First, both goldfish ovarian extracts, and porcine inhibin and activin are stimulatory to goldfish GTH-II release, and their actions are independent of DA inhibition. Second, goldfish ovarian extract prepared from recrudescing ovaries (CGOE-rec) has significantly higher potency in stimulating GTH-II release compared to those from regressed and mature ovaries. Third, pituitaries from sexually recrudescing goldfish exhibit significantly higher responsiveness to the ovarian extract from recrudescing ovaries (CGOE-rec). Fourth, the expression of messenger RNA for goldfish activin β B subunit significantly increases when the ovary enters the stage of recrudescence. Fifth, the cytoplasm of previtellogenic oocytes contains strong immunoreactivities with anti-inhibin and activin subunits; however, these immunoreactivities start to decrease once the oocytes enter the stage of recrudescence and eventually disappear completely when the oocytes become mature, perhaps due to the release of stored inhibin and activin molecules which are stimulatory to GTH-II release. Although this remains speculative, a hypothesis to test is that activin and probably inhibin

as well are produced in the goldfish ovary and sequestered in the previtellogenic oocytes. When the ovary begins to undergo recrudescence and some oocytes begin to accumulate cortical alveoli and yolk vesicles, the oocytes may process and release the stored activin and inhibin. Meanwhile, the ovarian follicles, in particular the follicle cells, increase the gene expression and production of these proteins. Activin and inhibin, from storage in the previtellogenic oocytes and newly synthesized, are released and transported to the pituitary to stimulate GTH-II release, thus contributing to the increasing levels of GTH-II during ovarian recrudescence; GTH in turn is necessary for vitellogenesis and the growth of oocytes. This hypothetical interpretation is diagrammatically represented in Fig.7.2. Considering that the actions of CGOE-rec and porcine inhibin and activin are independent of DA inhibition, and that CGOE-mat, and inhibin- and activin-containing pFF significantly potentiate sGnRH-stimulated GTH-II response, this stimulatory pathway may play important roles in breaking the strong tonic inhibitory tone by hypothalamic DA and, together with GnRH, initiate and maintain gonadal recrudescence. The strong desensitizing effects exhibited by CGOE-rec and pFF may provide a mechanism for these factors to restrain their own actions, and the maturation and ovulation of oocytes may automatically terminate the positive feedback. This hypothesis implies that compared to the ovary in mammalian species, goldfish ovary may participate actively in a positive feedback loop in driving ovarian development, together with the hypothalamus.

The more active and independent role of gonads in reproductive regulation in lower vertebrates is understandable in view of the fundamental metabolic difference between mammals and lower vertebrates such as fish. Mammals are endothermic animals the gonads of which, especially the ovaries, are essentially situated in a stable environment that is effectively isolated from the outside environment. Most, if not all, of the environmental cues that regulate reproductive functions have to be perceived and coordinated by central nervous system and reach gonads via mediation of the pituitary or

direct neural innervation, which places gonads, especially the ovaries, under a tight control by the brain. However, fish are ectothermic animals, and their gonads can be directly influenced by some environmental cues such as temperature without central nervous system and pituitary mediation. The environment-induced changes of gonadal activity may in turn influence the activities of the hypothalamus and pituitary, which coordinate the reproductive process. Some lines of evidence have emerged from studies on ectothermic species that support this concept: (1) The steroidogenesis in cultured turtle gonadal tissues shows extremely high sensitivity to temperature (Licht *et al.* 1989); (2) In a cyprinid fish, *Gnathopogon caeruleus*, high water temperature can directly act at the gonadal level to terminate the breeding season without affecting plasma GTH levels (Okuzawa *et al.*, 1989); (3) In common carp, *Cyprinus carpio* L., the ovarian steroidogenesis is dramatically influenced by temperature both *in vivo* and *in vitro*. It was proposed that the environmental temperature can regulate the reproductive cycle by directly acting on the ovary (Manning and Kime, 1984).

Clearly, these discussions remain largely hypothetical and the hypothesis proposed above is speculative, but they point to some directions for future research. Before concluding my thesis, I would like to list some of the questions that need to be addressed in the future.

(1) Are activin and inhibin subunits expressed in the oocytes? How are the gene expression and production of goldfish activin and inhibin molecules regulated in the gonads?

(2) Are goldfish gonadal activin and probably inhibin released from the gonads?

(3) What are the plasma profiles of these molecules and how are they related to the reproductive cycle?

(4) What are the second messenger pathways mediating the stimulatory actions of inhibin and activin on goldfish GTH-II and GH release?

(5) How are goldfish activin receptors compared to those in mammals?

(6) Do inhibin and activin bind the same receptors or do they each have distinct receptors?

Studies on these questions are feasible. With the amino acid sequences of goldfish activin subunits available now, oligopeptides can be synthesized and specific antibodies raised which can be used to establish specific radioimmunoassays for these subunits. The radioimmunoassays could be used to monitor plasma levels of these peptides and to study their production and regulation both *in vivo* and *in vitro*. Studies on the gene expression and regulation can be carried out using our cloned goldfish activin subunit genes as probes. A few activin receptors have been cloned and sequenced in mammals and *Xenopus* (Kondo *et al.*, 1991; Mathews and Vale, 1991; Matzuk and Bradley, 1992a, b) and the sequence information could be used to clone goldfish activin receptor genes. Considering the high structural conservation of activin subunits, there should be no major technical problems in this regard. Cloned goldfish activin receptor cDNA can be expressed in cultured cells that can be used to perform receptor binding assays (Mathews and Vale, 1991). The second messenger systems mediating stimulated release of GTH-II and GH have been better studied in goldfish than in any other teleostean species (Chang *et al.*, 1993), which will greatly benefit the investigation of the second messenger mechanisms of inhibin and activin actions on goldfish GTH-II and GH secretion. One of the problems in the present research has been the limitation of inhibin and activin supply. Given more inhibin and activin available, more detailed *in vitro* and *in vivo* studies have to be carried out in goldfish regarding their actions on GTH-II and GH secretion, which I believe will greatly expand our understandings of this exciting family of hormones in goldfish. The ultimate solution to this problem may be producing recombinant goldfish activin, which is also now technically feasible.

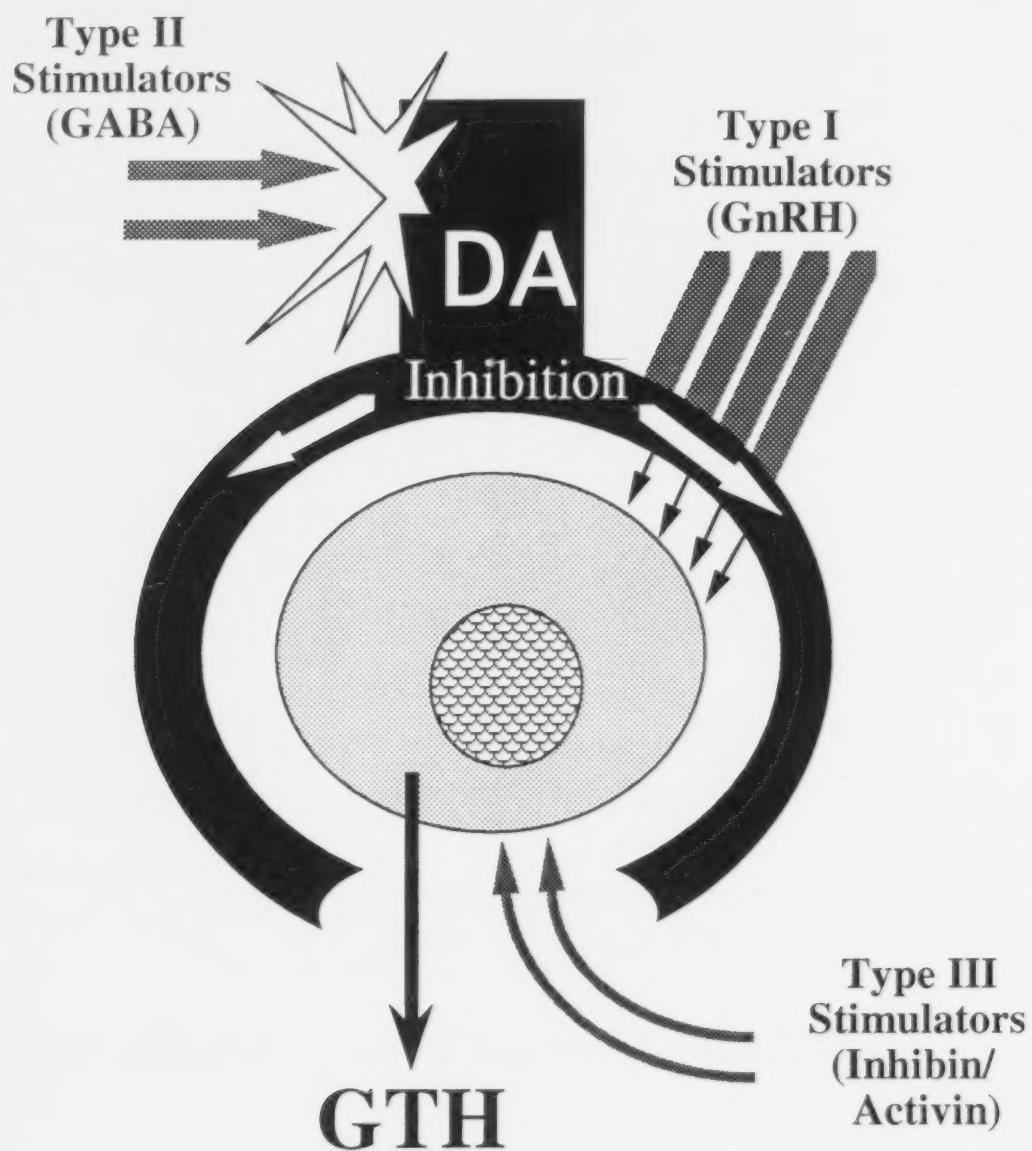


Fig.7.1. Diagrammatic representation of three major types of GTH stimulators in goldfish based on their relations with the hypothalamic dopamine tonic inhibition.

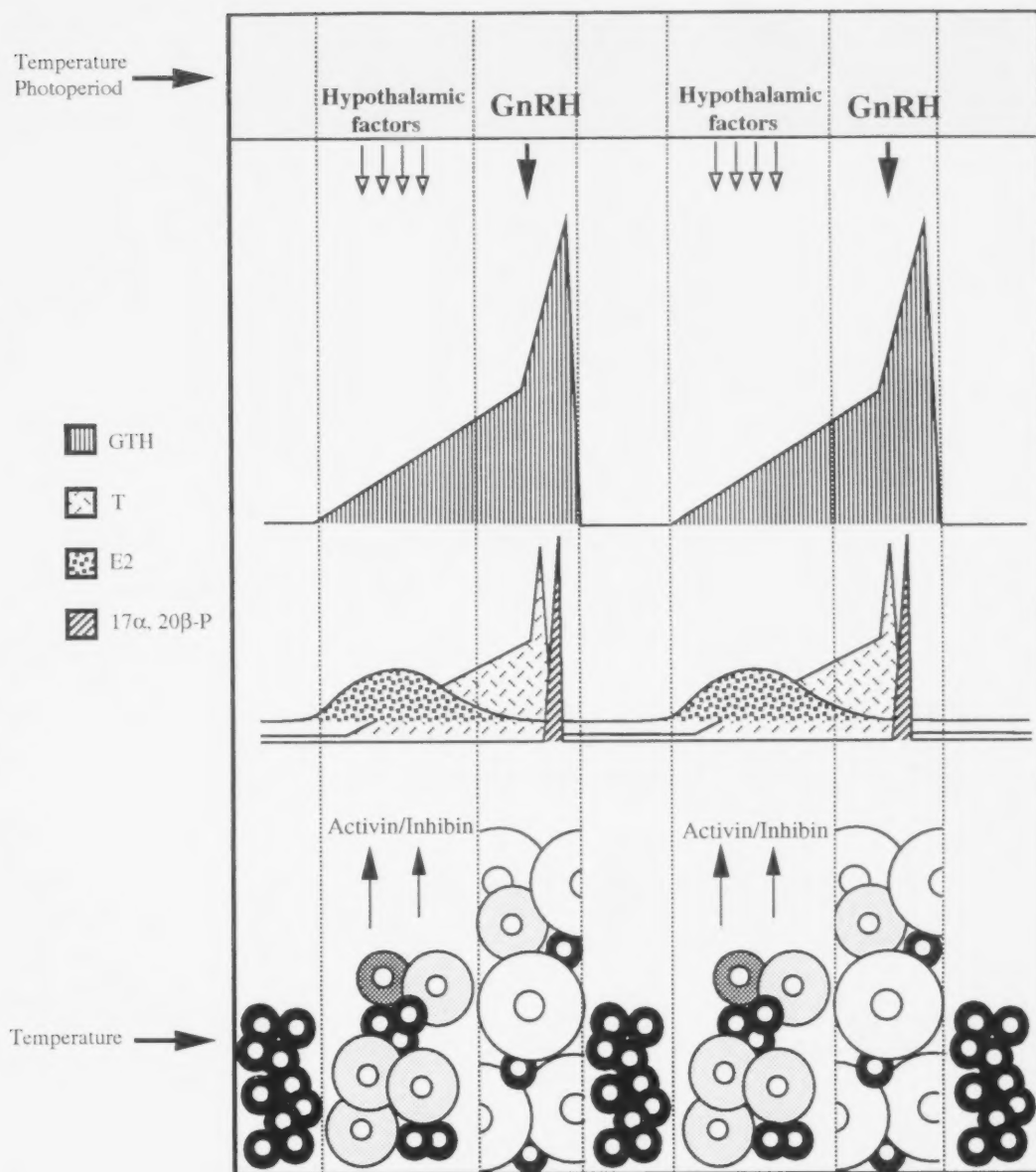


Fig. 7.2. Hypothetical involvement of ovarian activin/inhibin in the regulation of gonadotropin release. GTH, gonadotropin; T, testosterone; E2, estradiol; 17α, 20β-P, 17α, 20β-dihydroxy-4-pregnen-3-one. The hormone profiles are based on Kobayashi et al. (1988).

References

- Campbell, B.K., Mann, G.E., McNeilly, A.S. and Baird, D.T. 1990 The pattern of ovarian inhibin, estradiol, and androstenedione secretion during the estrous cycle of the ewe. *Endocrinology* 127:227-235
- Chang, J.P. and Peter, R.E. 1983 Effects of pimozone and Des Gly¹⁰, [D-Ala⁶] luteinizing hormone-releasing hormone ethylamide on serum gonadotropin concentrations, germinal vesicle migration, and ovulation in female goldfish, *Carassius auratus*. *Gen Comp Endocrinol* 52:30-37
- Chang, J.P., Jobin, R.M. and Wong, A.O.L. 1993 Intracellular mechanisms mediating gonadotropin and growth hormone release in the goldfish, *Carassius auratus*. *Fish Physiol Biochem* (in press)
- Chang, J.P., Van Goor, F. and Acharya, S. 1991 Influences of norepinephrine, and adrenergic agonists and antagonists on gonadotropin secretion from dispersed pituitary cells of goldfish, *Carassius auratus*. *Neuroendocrinology* 51:202-210
- Conn, P.M., Huckle, W.R., Andrews, W.V. and McArdle, C.A. 1987 The molecular mechanism of action of gonadotropin releasing hormone (GnRH) in the pituitary. *Rec Prog Horm Res* 43:29-67
- Corrigan, A.Z., Bilezikjian, L.M., Carroll, R.S., Bald, L.N., Schmelzer, C.H., Fendly, B.M., Mason, A.J., Chin, W.W., Schwall, R.H. and Vale, W. 1991 Evidence for an autocrine role of activin B within rat anterior pituitary cultures. *Endocrinology* 128:1682-1684
- Cuevas, P., Ying, S-Y., Ling, N., Esch, F. and Guillemin, R. 1987 Immunohistochemical detection of inhibin in the gonad. *Biochem Biophys Res Commun* 142:23-30
- DePaolo, L.V. 1991 Hypersecretion of follicle-stimulating hormone (FSH) after ovariectomy of hypophysectomized, pituitary-grafted rats: implication for local regulatory control of FSH. *Endocrinology* 128:1731-1740
- Dulka, J.G., Sloley, B.D., Stacey, N.E. and Peter, R.E. 1992 A reduction in pituitary dopamine turnover is associated with sex pheromone-induced gonadotropin secretion in male goldfish. *Gen Comp Endocrinol* 86:496-505
- Everett, J.W. and Nikitovitch-Winer, M. 1963 Physiology of the pituitary gland as affected by transplantation or stalk section. In Nalbandov, A.V. (ed): *Advances in Neuroendocrinology*. University of Illinois Press, Urbana. pp. 289-304
- Findlay, J.K., Clarke, I.J. and Roberston, D.M. 1990 Inhibin concentrations in ovarian and jugular venous plasma and the relationship of inhibin with follicle-stimulating hormone and luteinizing hormone during the ovine estrous cycle. *Endocrinology* 126:528-535
- Gharib, S.D., Wierman, M.E., Shupnik, M.A. and Chin, W.W. 1990 Molecular biology of the pituitary gonadotropins. *Endocr Rev* 11:177-199

biology of the pituitary gonadotropins. *Endocr Rev* 11:177-199

- Haisenleder, D.J., Ortolano, G.A., Dalkin, A.C., Landefeld, T.D., Vale, W.W. and Marshall, J.C. 1990 Inhibin secretion during the rat estrous cycle: relationships to FSH secretion and FSH beta subunit mRNA concentrations. *Life Sci* 47:1769-1773
- Hasegawa, Y., Miyamoto, K., Iwamura, S. and Igarashi, M. 1988 Changes in serum concentrations of inhibin in cyclic pigs. *J Endocrinol* 118:211-219
- Idler, D.R. and Ng, T.B. 1983 Teleost gonadotropins: isolation, biochemistry, and function. In *Fish physiology* Vol. IX. Hoar, W.S., D.J. Randall, and E.M. Donaldson. (eds). Academic Press, New York. pp. 187-221
- Kah, O., Trudeau, V.L., Sloley, B.D., Chang, J.P., Dubourg, P., Yu, K.L. and Peter, R.E. 1992 Influence of GABA on gonadotropin release in the goldfish. *Neuroendocrinology* 55:396-404
- Karsch, F.J. 1987 Central actions of ovarian steroids in the feedback regulation of pulsatile secretion of luteinizing hormone. *Ann Rev Physiol* 49:365-382
- Kobayashi, M., Aida, K. and Hanyu, I. 1988 Hormone change during the ovulatory cycle in goldfish. *Gen Comp Endocrinol* 69:301-307
- Kobayashi, M. and Stacey, N.E. 1990 Effects of ovariectomy and steroid hormone implantation on serum gonadotropin levels in female goldfish. *Zool Sci* 7:715-721
- Kondo, M., Tashiro, K., Fujii, G., Asano, M., Miyoshi, R., Yamada, R., Muramatsu, M. and Shiokawa, K. 1991 Activin receptor mRNA is expressed early in *Xenopus* embryogenesis and the level of the expression affects the body axis formation. *Biochem Biophys Res Commun* 181:684-690
- Lazier, C.B., Mann, M. and Mommsen, T.P. 1987 Estrogen receptor and regulation of vitellogenesis. In *Proceedings of the Third International Symposium on the Reproductive Physiology of Fish*, St. John's, Newfoundland, Canada, 2-7 August 1987. pp.178-182
- Licht, P., Denver, R.J. and Pavgi, S. 1989 Temperature dependence of *in vitro* pituitary, testis, and thyroid secretion in a turtle, *Pseudemys scripta*. *Gen Comp Endocrinol* 76:274-285
- Manning, N.J. and Kime, D.E. 1984 Temperature regulation of ovarian steroid production in the common carp, *Cyprinus carpio* L., *in vivo* and *in vitro*. *Gen Comp Endocrinol* 56:376-388
- Marchant, T.A., Chang, J.P., Nahorniak, C.S. and Peter, R.E. 1989 Evidence that gonadotropin-releasing hormone also functions as a growth hormone-releasing factor in the goldfish. *Endocrinology* 124:2509-2518
- Mathews, L.S. and Vale, W.W. 1991 Expression cloning of an activin receptor, a predicted transmembrane serine kinase. *Cell* 65:973-982

- Matzuk, M.M. and Bradley, A. 1992a Structure of the mouse activin receptor type II gene. *Biochem Biophys Res Commun* 185:404-413
- Matzuk, M.M. and Bradley, A. 1992b Cloning of the human activin receptor cDNA reveals high evolutionary conservation. *Biochimica et Biophysica Acta* 1130:105-108
- McDonald, J.K., Lumpkin, M.D., Samson, W.K. and McCann, S.M. 1985 Neuropeptide Y affects secretion of luteinizing hormone and growth hormone in ovariectomized rats. *Proc Natl Acad Sci USA* 82:561-564
- McLachlan, R.I., Dahl, K.D., Bremner, W.J., Schwall, R., Schmelzer, C.H., Mason, A.J. and Steiner, R. 1989 Recombinant human activin-A stimulates basal FSH and GnRH-stimulated FSH and LH release in the adult male macaque, *Macaca fascicularis*. *Endocrinology* 125:2787-2789
- Merchenthaler, I., Celler, M.D., Petrusz, P. and Negro-Vilar, A. 1987 Immunocytochemical localization of inhibin in rat and human reproductive tissues. *Mol Cell Endocrinol* 54:239-243
- Meunier, H., Rivier, C., Evans, R.M. and Vale, W. 1988 Gonadal and extragonadal expression of inhibin α , β A, and β B subunits in various tissues predicts diverse functions. *Proc Natl Acad Sci USA* 85:247-251
- Michel, U., Jarry, H., Metten, M. and Wuttke, W. 1989 Inhibin production by porcine granulosa and luteal cells: development and biological validation of a RIA. *Acta Endocrinol (Copen)* 120:511-518
- Negro-Vilar, A., Ojeda, S.R. and McCann, S.M. 1979 Catecholaminergic modulation of luteinizing hormone-releasing hormone release by median eminence terminals *in vitro*. *Endocrinology* 104:1749-1757
- Ng, T.B. and Idler, D.R. 1983 Yolk formation and differentiation in teleost fishes. In: *Fish physiology* Vol. IX. (Hoar, W.S., Randall, D.J. and Donaldson, E.M. eds.). Academic Press, New York. pp. 373-404
- Okuzawa, K., Furukawa, K., Aida, K. and Hanyu, I. 1989 Effects of photoperiod and temperature on gonadal maturation, and plasma steroid and gonadotropin levels in cyprinid fish, the honmoroko *Gnathopogon caeruleus*. *Gen Comp Endocrinol* 75:139-147
- Omeljaniuk, R.J., Shih, S.H. and Peter, R.E. 1986 *In vivo* evaluation of dopamine receptor-mediated inhibition of gonadotrophin secretion from the pituitary gland of the goldfish, *Carassius auratus*. *J Endocrinol* 114:449-458
- Peng, C., Huang, Y-P. and Peter, R.E. 1990 Neuropeptide Y stimulates growth hormone and gonadotropin release from the goldfish pituitary *in vitro*. *Neuroendocrinology* 52:28-34
- Peter, R.E. 1981 Gonadotropin secretion during reproductive cycles in teleosts:

- influences of environmental factors. *Gen Comp Endocrinol* 45:294-305
- Peter, R.E. and Crim, L.W. 1979 Reproductive endocrinology of fishes: gonadal cycles and gonadotropin in teleosts. *Ann Rev Physiol* 41:323-335
- Peter, R.E., Chang, J.P., Nahorniak, C.S., Omeljaniuk, R.J., Sokolowska, M., Shih, S.H. and Billard, R. 1986 Interaction of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. *Rec Prog Horm Res* 42:513-547
- Peter, R.E., Nahorniak, C.S., Chang, J.P. and Crim, L.W. 1984 Gonadotropin release from the pars distalis of goldfish, *Carassius auratus*, transplanted beside the brain or into the brain ventricles: additional evidence for gonadotropin-releasing-inhibitory factor. *Gen Comp Endocrinol* 55:337-346
- Rivier, C. and Vale, W. 1987 Inhibin: measurement and role in the immature female rat. *Endocrinology* 120:1688-1690
- Roberts, V., Meunier, H., Vaughan, J., Rivier, J., Rivier, C., Vale, W. and Sawchenko, P. 1989 Production and regulation of inhibin subunits in pituitary gonadotropes. *Endocrinology* 124:552-554
- Shintani, Y., Takada, Y., Yamasaki, R. and Saito, S. 1991 Radioimmunoassay for activin/EDF: method and measurement of immunoreactive activin A/EDF levels in various biological materials. *J Immunol Meth* 137:267-274
- Trudeau, V.L., Murthy, C.K., Habibi, H.R., Sloley, B.D. and Peter, R.E. 1993 Effects of sex steroid treatments on gonadotropin-releasing hormone-stimulated gonadotropin secretion from the goldfish pituitary. *Biol Reprod* 48:300-307
- Trudeau, V.L., Peter, R.E. and Sloley, B.D. 1991 Testosterone and estradiol potentiate the serum gonadotropin response to gonadotropin-releasing hormone in goldfish. *Biol Reprod* 44:951-960
- Trudeau, V.L., Sloley, B.D. and Peter, R.E. 1993 GABA stimulation of gonadotropin-II release in the goldfish: involvement of GABAA receptors, dopamine and sex steroids. (submitted)
- Vale, W., Rivier, C., Hsueh, A., Campen, C., Meunier, H., Bicsak, T., Vaughan, J., Corrigan, A., Bardin, W., Sawchenko, P., Petraglia, F., Yu, J., Plotsky, P., Spiess, J. and Rivier, J. 1988 Chemical and biological characterization of the inhibin family of protein hormones. *Recent Prog. Horm Res* 44:1-34
- Wong, A.O.L., Chang, J.P. and Peter, R.E. 1992 Dopamine stimulates growth hormone release from the pituitary of goldfish, *Carassius auratus*, through the dopamine D1 receptors. *Endocrinology* 130:1201-1210
- Ying, S.Y. 1988a Inhibins, activins, and follistatins: gonadal proteins modulating the secretion of follicle-stimulating hormone. *Endocr Rev* 9:267-293
- Ying, S.Y. 1988b Inhibins and activins. In: *Frontiers in Neuroendocrinology*. (Martini, L. and Ganong, W.F., eds.). Raven Press, Ltd. New York. 10:167-184

- Yu, K.L., Peng, C. and Peter, R.E. 1989 Changes in brain levels of gonadotropin-releasing hormone and serum levels of gonadotropin and growth hormone in goldfish during spawning. *Can J Zool* 69:182-188
- Zhang, Z., Carson, R., Herington, A., Lee, V. and Burger, H.G. 1987 Follicle stimulating hormone and somatomedin C stimulate inhibin production by rat granulosa cells *in vitro*. *Endocrinology* 120:1633-1638

Appendix

Chapter Eight

IMMUNOREACTIVE ACTIVIN SUBUNITS IN THE GOLDFISH BRAIN

Introduction

Inhibin and activin subunits have been shown to have widespread distribution in a variety of extragonadal tissues (Meunier *et al.*, 1988) and play paracrine and autocrine roles in regulating many physiological activities (Chen, 1993). All three inhibin and activin subunit mRNAs (α , β A and β B) have been shown to be expressed in the rat central nervous system (Meunier *et al.*, 1988), suggesting that these proteins have actions in the nervous system. The gene expression of inhibin and activin subunits in the nervous system has further been confirmed by immunocytochemical stainings for β A and β B (Sawchenko *et al.*, 1988). The immunoreactive nerve terminals have been found in the septal-preoptic region, and the paraventricular (PVN) and supraoptic (SON) nuclei where neurons containing gonadotropin-releasing hormone and oxytocin are located, respectively (Sawchenko *et al.*, 1988). These findings are in agreement with the reported biological actions of inhibin and activin in the brain. Inhibin appears to exert an inhibitory action on rat hypothalamic GnRH release (de Greef *et al.*, 1983; Demoulin *et al.*, 1979). Injection of activin into the PVN stimulates oxytocin secretion in the rat, and infusion of anti- β sera in the PVN suppresses suckling-induced oxytocin release, suggesting that activin may be involved in the mediation of suckling-induced oxytocin secretion (Sawchenko *et al.*, 1988). These results indicate that brain activin, and

probably inhibin as well, are involved in the regulation of some aspects of reproduction. In addition to the biological effects mentioned above, activin and inhibin have been demonstrated to promote the survival of certain nerve cell lines cultured *in vitro* (Schubert *et al.*, 1990).

To examine if the immunoreactive activin and inhibin subunits may exist in the goldfish brain, we carried out a preliminary study on the presence and distribution of activin and inhibin subunits βA and βB by immunocytochemistry.

Materials and Methods

Animals and tissue preparation

Female goldfish (*Carassius auratus*) were used in the present study. Animals were anesthetized with 0.05% tricaine methanesulphonate before handling.

The brains were removed from the goldfish and immediately fixed in Bouin's fixative at 4°C for 24 hours, followed by washes with 70% ethanol until the picric acid was washed away. After dehydration through a graded series of ethanol solutions, the tissues were embedded in paraffin. The whole brain was cut serially at 10 μm and mounted in consecutive order on poly-L-lysine coated slides for immunostaining.

Antibodies

Affinity purified domain-specific antibodies, rabbit anti-porcine inhibin βA (81-113)-NH₂ (Code #160-209-A) and anti-human inhibin βB (80-112)-NH₂ (Code #160-213-A), were obtained from Drs. W. Vale and J. Vaughan, Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA, USA. Each antiserum was produced in rabbits against synthetic inhibin fragments coupled to human α -globulins. Specific antibodies were affinity-purified by solid phase

absorption to the corresponding peptide, eluted, neutralized, filtered and diluted in storage buffer. Preparation and characterization of the antisera have been described in detail previously (Meunier et al., 1988; Roberts et al., 1989; Vaughan et al., 1989).

Immunocytochemistry

Sections were deparaffinized, hydrated and immunocytochemically stained using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) based on the avidin-biotin-peroxidase complex (ABC) method (Hsu et al., 1981) with some modifications. Briefly, the sections were incubated in 0.3% H₂O₂ in methanol for 40 minutes to deactivate endogenous peroxidase activity and washed with 10 mM potassium phosphate buffered saline (PBS, 0.15 M NaCl, pH 7.5) for 20 minutes followed by 20 minute incubation with 0.5% gelatin in PBS. After incubation with normal goat serum (1:40 dilution) for 30 minutes, the sections were incubated for 24 hours at 4°C in a humidified chamber with the primary antibodies against inhibin subunits diluted with PBS at 1:100 for anti- α , and 1:200 for anti- β A and anti- β B. After the sections were treated with the biotinylated goat anti-rabbit antibody for 60 minutes, the ABC reagent in PBS (0.5 M NaCl, pH 7.5) was applied for 60 minutes; 0.05% diaminobenzidine and 0.01% H₂O₂ were used as substrates. The sections were then rinsed, dehydrated and mounted for microscopic observation.

Results and Discussion

One nucleus in goldfish brain showed strong and equal immunoreactivity with anti-activin β A and β B (Fig. 8.1a and Fig. 8.2). This nucleus was identified as nucleus pretectalis rotundus (NPR) according to Peter and Gill (1975). The immunoreactive neurons can be clearly seen in the nucleus and the bundles of fibres that project out from

the nucleus are also strongly stained by anti-activin β A and β B (Fig. 8.1b, c and d). The destination of the fibres is not clear. In addition to NPR, a group of immunoreactive neurons are also found in the nucleus anterior tuberis (NAT) (Fig. 8.1a and Fig. 8.2).

Since the functions of nuclei NPR and NAT in goldfish are not clear, it is hard to predict any potential physiological significance of the immunoreactive activin subunits in these nuclei. Some giant neuron cell bodies in the preoptic region of the hypothalamus were also stained, but the stainings were too weak to judge (data not shown). The present study indicates that goldfish activin and perhaps inhibin as well are expressed and produced in the goldfish brain, and may exert biological actions within the central nervous system as in mammalian species.

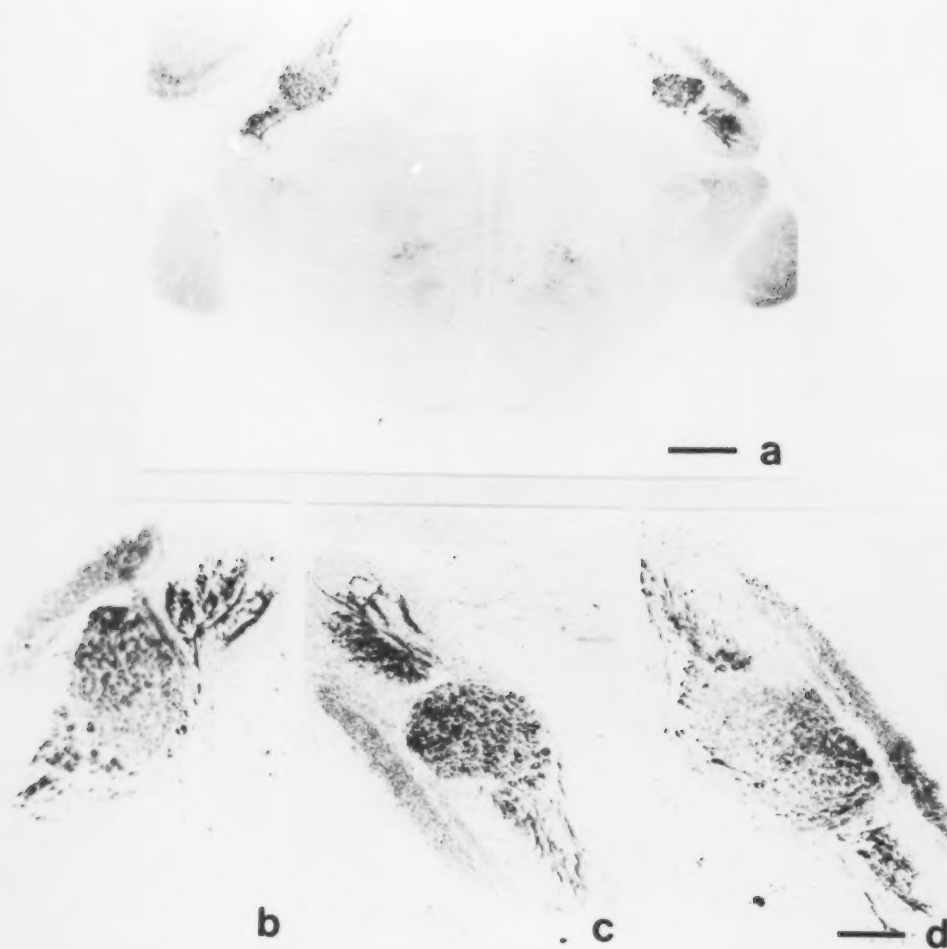
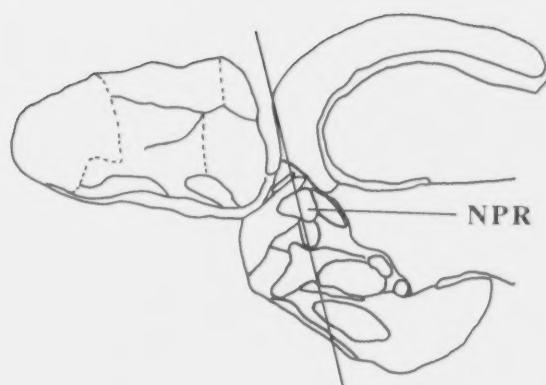


Fig. 8.1. Immunocytochemical staining for activin and inhibin β B (a and c) and β A (b and d) subunits in the goldfish brain. Bar for a: 105 μ m; bar for b, c and d: 45 μ m.

a



b

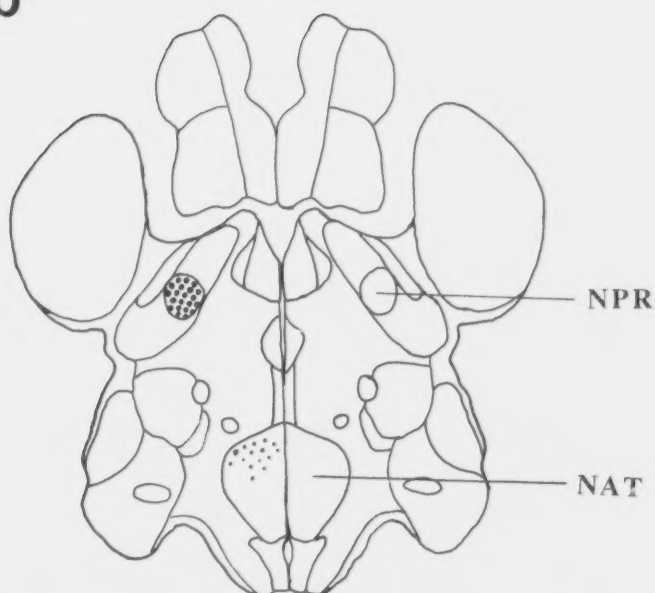


Fig. 8.2. Diagrammatic representation of activin and inhibin β A and β B immunoreactive areas in the goldfish brain. (a) Section orientation; (b) Diagrammatic localization of the immunoreactive nuclei. NPR, nucleus pretectalis rotundus; NAT, nucleus anterior tubercis.

References

- Chen, C-L.C. 1993 Editorial: inhibin and activin as paracrine/autocrine factors. *Endocrinology* 132:4-5
- de Greef, W.J., de Jong, F.H., de Koning, J., Steenbergen, J. and Van der Vaart, P.D.M. 1983 Studies on the mechanism of the selective suppression of plasma levels of follicle-stimulating hormone in the female rat after administration of steroid-free bovine follicular fluid. *J Endocrinol* 97:327-338
- Demoulin, A., Bourguignon, J.P. and Franchimont, P. 1979 "In vitro" inhibition of hypothalamic LH-RH by human inhibin. *Acta Endocrinol Suppl* 225:227
- Hsu, S.M., Raine, L. and Fanger, H. 1981 Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem and Cytochem* 29:577-580
- Meunier, H., Rivier, C., Evans, R.M. and Vale, W. 1988 Gonadal and extragonadal expression of inhibin α , β A, and β B subunits in various tissues predicts diverse functions. *Proc Natl Acad Sci USA* 85:247-251
- Peter, R.E. and Gill, V.E. 1975 A stereotaxic atlas and technique for forebrain nuclei of the goldfish, *Carassius auratus*. *J Comp Neur* 159:69-102
- Roberts, V., Meunier, H., Sawchenko, P.E. and Vale, W. 1989 Differential production and regulation of inhibin subunits in rat testicular cell types. *Endocrinology* 125:2350-2359
- Sawchenko, P.E., Plotsky, P.M., Pfeiffer, S.W., Cunningham, E.T. Jr, Vaughan, J., Rivier, J. and Vale, W. 1988 Inhibin β in central neural pathways involved in the control of oxytocin secretion. *Nature* 334:615-617
- Schubert, D., Kimura, H., LaCorbiere, M., Vaughan, J., Karr, D. and Fisher, W.H. 1990 Activin is a nerve cell survival molecule. *Nature* 344:868-870
- Vaughan, J.M., Rivier, J., Corrigan, A.Z., McClintock, R., Campen, C.A., Jolley, D., Voglmayr, J.K., Bardin, C.W., Rivier, C. and Vale, W. 1989 Detection and purification of inhibin using antisera generated against synthetic peptide fragments. In: *Neuroendocrine Peptides, Methods in Enzymology* Vol 168. (Conn, P.M., ed.). Academic Press, Orlando. pp. 588-617

Appendix

Chapter Nine

PURIFICATION, PARTIAL MICROSEQUENCING AND CHARACTERIZATION OF GOLDFISH GONADAL TRANSFERRIN

Introduction

Vertebrate gonads produce a wide range of substances including steroids, peptides and proteins, many of which are involved in the intragonadal regulation of gonadal hormone production, gametogenesis and maintenance of mature gametes. Unlike mammalian follicular fluid, goldfish ovarian fluid has fewer protein components when examined on SDS-PAGE. Interestingly, goldfish seminal plasma has similar protein compositions to those of ovarian fluid. One of the major proteins of goldfish ovarian fluid and seminal plasma was also identified and concentrated during the course of studies on goldfish activin purification from ovarian extract. The reduced form of this protein had slower mobility than its non-reduced form on SDS-electrophoresis. This peculiar behavior was used to further purify this protein from the ovarian extract and ovarian fluid. Its reduced but not non-reduced form could occasionally crossreact with anti-inhibin β B antibody for unknown reasons. This protein was eventually purified from the goldfish ovarian extract and identified to be transferrin using the technique of protein microsequencing.

There is no information available about fish transferrin. This chapter presents data about purification and identification of this goldfish gonadal transferrin.

Materials and Methods

Experimental Animals

Sexually mature goldfish (*Carassius auratus*) were purchased from Grassyfork Fisheries Co., Martinsville, IN, USA or Ozark Fisheries, Stoutland, MO, USA. Fish were acclimated to $17^{\circ}\text{C} \pm 1$ and a simulated natural photoperiod of Edmonton, and fed a commercial fish food for at least one week before use. Animals were anaesthetized with 0.05 % tricaine methanesulphonate before handling.

Crude goldfish ovarian extract (CGOE) preparation

Mature goldfish ovaries were homogenized with Polytron homogenizer and extracted in phosphate-buffered saline (PBS, 50 mM + 0.8% NaCl, pH 7.4) (1 wt.:1.5 vol.) containing 0.01 M phenylmethylsulfonyl fluoride (PMSF, a proteinase inhibitor) for 3 hours at 4°C . The homogenates were centrifuged for 1 hour at 20,000 g, and the supernatants were dialysed (with 12 kD cut-off) against double distilled water for 24 hours, lyophilized, and stored at -20°C for subsequent use.

Preparation of ovarian fluid and seminal plasma

Goldfish ovarian fluid and seminal plasma were collected by centrifuging the ovulated eggs and milt, respectively.

Fractionation of ovarian extract

To eliminate the large amount of yolk proteins in the goldfish ovarian extract, the extract was precipitated with ammonium sulphate of 60% saturation. The supernatant (AS-S) was dialysed in distilled water for 80 hours before lyophilization. The lyophilized samples were then dissolved in 0.02 M ammonium bicarbonate buffer (pH

9.0) and subject to ion exchange chromatography on DEAE Bio Gel A column using stepwise gradient elution. This yielded four fractions (DE-1, 2, 3 and 4), and each fraction was dialysed and lyophilized. The first fraction (DE-1) was then dissolved in 0.02 M ammonium bicarbonate (pH 9.0) and further separated on Sephadex G100 gel filtration column. Three fractions were generated (G 100 A, B and C). The fraction G 100 A was dissolved in 0.01 M Tris-HCl buffer (pH 8.5) and separated on DEAE BioGel A using continuous gradient elution (0-0.2 M NaCl). Two fractions were obtained (Fraction A and Fraction B). All the fractions from each step were examined by SDS electrophoresis.

Analytical SDS electrophoresis

Both Weber and Osborn's (1969) continuous and Laemmli's (1970) discontinuous SDS-PAGE were performed using Mini-PROTEIN II system (BioRad, Hercules, CA) to monitor the chromatographic procedures, with the polyacrylamide gel concentrations being 7.5% and 10% or 12.5%, respectively. The proteins in the gels were visualized with Coomassie R-250 or silver staining. BioRad's low range SDS-PAGE Standards was used for measurement of the molecular size (BioRad, Hercules, CA).

Preparative SDS electrophoresis

The final step of purification was performed using preparative SDS electrophoresis of Laemmli's system. Briefly, 7.5% polyacrylamide gel was casted with the preparative well formed using a 2-D/Preparative comb (BioRad, Hercules, CA). About 800 µg of lyophilized sample of Fraction B was dissolved and treated in 200 µl non-reducing sample buffer at 37°C for 15 minutes, and subject to electrophoresis. After the electrophoresis, the slices at both edges of the gel were cut off and stained with

Coomassie R-250. The stained edges were then aligned with the rest of the gel to locate the desired protein band. The protein band was cut into small pieces of about 2 mm and eluted in 0.1% SDS solution at 37°C three times for 24 hours each. The extracts were pooled and lyophilized. To remove the SDS, the lyophilized sample was dissolved in 50 µl 0.1 M ammonium bicarbonate (pH 7.4) containing 8 M urea with 0.05% phenol red as marker. The sample was then loaded in a Sephadex G25 gel filtration column (0.7 X 8 cm). The second ml of the eluent was collected, and 30 µl of the collected eluent was examined on SDS-PAGE. The rest of the sample was lyophilized.

Western blot analysis

Protein samples were electrophoresed on Laemmli's SDS-PAGE and electroblotted onto nitrocellulose membrane overnight in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3). The blot was blocked in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) containing 4% gelatin and 0.1% NaN₃ for 2 hours followed by three washes in TTBS (20 mM Tris, 500 mM NaCl, 0.05% Tween 20, pH 7.5). The blot was then subject to incubation for 20 hours with primary antibodies at dilutions of 1:250 and 1:500 for anti-inhibin βA and βB, respectively. After treatment with the second antibody and alkaline phosphatase conjugate (BioRad, Hercules, CA) at a dilution of 1:1500, the immunoreactive protein bands were visualized with substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT).

High performance liquid chromatography (HPLC)

The purity of eluent from SDS-PAGE gel was examined by reversed-phase HPLC. 2 µg of the sample was loaded to Aquapore RP-300 (C8, 300 Å pore size, 7 µm particle size) column (1 X 100 mm; Brownlee Labs, San Jose, CA). The column was eluted with a linear gradient of 0-100% acetonitrile (2% acetonitrile/minute) in 0.05%

trifluoroacetic acid (TFA) at the flow rate of 0.1 ml/minute.

Amino acid analysis

10 µg purified protein was hydrolyzed with 6 N HCl for 24 hours at 110°C, and then subject to automated amino acid analysis on Beckman 6300 Amino Acid Analyzer (Beckman, Palo Alto, CA) based on the ion exchange-ninhydrin method (Ozols, 1990).

Microsequencing

Microsequencing of amino acids was according to Fischer et al. (1991). Briefly, 20 µg of purified protein sample was reconstituted in 50 µl water. 10 µl of the dissolved sample was run on an 8% SDS mini-gel and then electroblotted to a PVDF membrane in CAPS transfer buffer (10 mM CAPS, 10% methanol, pH 11) using a BioRad Mini Trans-Blot system (BioRad, Hercules, CA). Protein blot was stained with 0.1% Ponceau S containing 10% methanol, 1.5% trichloroacetic acid and 1.5% sulfosalicylic acid. The protein band was excised and the N-terminal amino acid sequence was determined using an Applied Biosystems Protein Sequencer 470A (Applied Biosystems, Foster City, CA). The remainder of the sample (40 µl) was also run on an 8% SDS mini-gel and electroblotted to a PVDF membrane. The protein band was excised and subject to *in situ* digestion with 1 µg/ml trypsin (sequencing grade, Boehringer Mannheim Biochemicals, Indianapolis, IN) overnight at 37°C. The peptide fragments generated by the digestion were separated by reversed phase HPLC (column: Vydac C18, 2.1X100 mm, 5 µm particle size). Two fractions (fraction 25 and 67) were subject to sequence analysis.

Results

1. SDS electrophoretic analysis of goldfish ovarian fluid and seminal plasma

The goldfish ovarian fluid and seminal plasma were collected and subject to SDS-PAGE analysis. The number of protein bands revealed was much less than porcine follicular fluid (pFF) (data not shown). Interestingly, the female and male gonadal fluids show similar patterns of protein components (Fig. 9.1). One of the dominant proteins of about 57 kD was interesting in that after being reduced with β -mercaptoethanol, it was occasionally found to cross-react with anti-inhibin β B antibody in Western blot analysis, and its mobility in electrophoresis became significantly lower after reduction (70 kD). This shift in migration rate on SDS-PAGE was later used to help monitor the purification of this protein.

2. Purification of the 57 kD protein from goldfish ovarian extract (CGOE-mat)

Since the amounts of either goldfish ovarian fluid or seminal plasma were very limited, and the protein described above was also identified in ovarian extracts, the purification procedures were carried out using the ovarian extracts from mature ovaries. One of the problems with ovarian extracts is the large amount of yolk proteins. The first step of precipitation with ammonium sulfate effectively removed most of the yolk proteins, and after ion exchange chromatography and gel filtration, the 57 kD protein and one other protein were significantly concentrated (Fig. 9.2). The sample from the gel filtration step was further fractionated by ion exchange chromatography using a different buffer system and elution pattern. The 57 kD protein was effectively separated from others after this step (Fig. 9.3). The final purification was performed using a preparative SDS electrophoresis which gave a single homogeneous band when examined on SDS-PAGE (Fig. 9.3). HPLC analysis showed that the protein was about 90% pure (Fig.

9.4).

3. Amino acid analysis and determination of amino acid sequences

N-terminal sequence analysis yielded the following sequence: Ala Gln (Asp, Pro) Val Lys - (Pro) Val Lys Ser Gln Asn

After digestion with trypsin, the peptide fragments were eluted from PVDF membrane and subject to HPLC separation. Two fractions (Fr. 25 and 67) were chosen and subject to sequence analysis (Fig. 9.6). The amino acid sequences yielded are:

Fr. 25: Val Pro Ala His Ala Val Ile Thr Arg

Fr. 67: (Gly) Gly Asp Ala Asp Ala Ile Thr Val Asp Ala Gly His Val Tyr Leu Ala Gly Leu

The amino acid compositions of the 57 kD protein were shown in Table 9.1.

5. Protein identification

The N-terminal amino acid sequence and the two internal sequences were subjected to GenBank database for sequence search via program BLASTP. The protein that exhibits highest identity with the 57 kD goldfish protein is transferrin. The sequence alignment is presented in Fig. 9.7.

Discussion

Transferrin is an iron-binding protein that is present in serum and various tissues (Aisen and Listowsky, 1980). Our attempts at identifying and isolating goldfish inhibin and activin-like molecules led to the purification of goldfish ovarian transferrin, which represents the first in fish. The occasional cross-reaction of the reduced but not non-reduced goldfish transferrin with anti-inhibin β B antibody in Western blot analysis is

probably due to non-specific adherence, since the reduction of the protein may expose its hydrophobic core which in turn may cause it to bind to antibody molecules. The lower mobility of the protein on SDS-PAGE after reduction suggests that goldfish transferrin contains intramolecular disulfide bonds, and that reduction causes conformational changes.

In mammals, serum transferrin plays an important physiological role in transporting iron to various tissues and has significant effects on cell proliferation *in vitro* (Aisen and Listowsky, 1980). Transferrin is required for cell growth in most serum-free culture systems (Barnes and Sato, 1980). Transferrin is now considered not only a iron-binding protein, but also an important growth factor in circulation to regulate cell proliferation and tissue differentiation *in vivo* (Beach *et al.*, 1983; Ekblom *et al.*, 1983; Levin *et al.*, 1984). Transferrin has been demonstrated to act as a neurotrophic factor (NTF) to promote [³H]thymidine incorporation and myotube formation in chick muscle cultures (Beach *et al.*, 1983), and transferrin mRNA has been detected in the rat brain and the level of gene expression increases during the postnatal development (Levin *et al.*, 1984); therefore, it was proposed that transferrin synthesized in the central nervous system may be transported through axon fibres to the muscle to function as a neurotrophic factor mediating the trophic actions of nervous system on the development of the muscle (Beach *et al.*, 1983; Levin *et al.*, 1984). Transferrin also plays a critical role in inducing kidney differentiation during mouse embryogenesis and no other serum factors can replace transferrin for this function (Ekblom *et al.*, 1983).

Transferrin has been demonstrated in mammalian testes including seminiferous tubule fluid and rete testis fluid (Sylvester and Griswold, 1984), and it has been purified from seminal plasma of a variety of mammalian species (for review see Shivaji *et al.*, 1990). Transferrin is the most abundant protein produced by Sertoli cells (Skinner and Griswold, 1980). Since high concentrations of iron appear to be necessary for the early

stages of spermatogenesis, and transferrin has been demonstrated to be associated with germ cells of early stages, the function of the testicular transferrin is believed to be transferring iron across the blood-testicular barrier and delivering it to the developing germ cells (Sylvester and Griswold, 1984). The levels of immunoreactive transferrin in the seminal plasma fluctuate significantly and show a close positive relatedness to the number of spermatozoa (Khalfoun *et al.*, 1986). The gene expression of testicular transferrin also significantly decreases with the depletion of germ cells in rats (Roberts *et al.*, 1992), suggesting a significant role for transferrin in the production and/or maintenance of the sperm. The production and gene expression of transferrin in Sertoli cells is stimulated by FSH, insulin, vitamin A (Huggenvik *et al.*, 1987), and the germ cells can also somehow directly increase the gene expression of transferrin in Sertoli cells (Roberts *et al.*, 1991).

Goldfish transferrin is present in both female ovarian fluid and male seminal plasma as one of the major protein components. The functions of transferrin in the gonadal fluids remain unknown. The abundance of transferrin in the ovarian and seminal plasma suggests that this protein may be involved in maintaining the vitality of the gametes. This study also represents the current technology for purifying and identifying novel proteins of potential physiological significance.

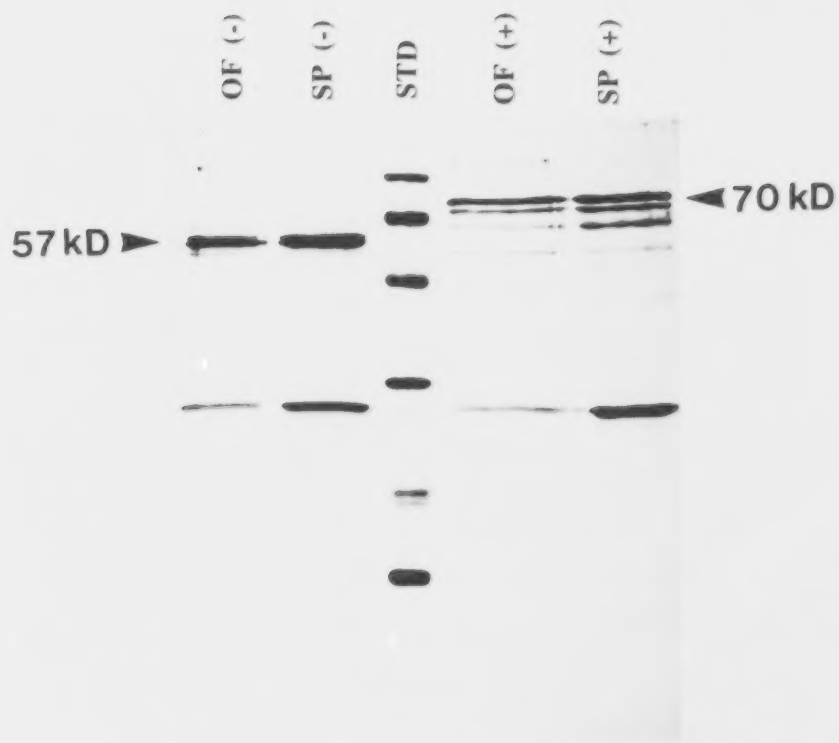


Fig. 9.1. SDS-PAGE analysis (12.5%) of goldfish ovarian fluid (OF) and seminal plasma (SP) under non-reducing (-) and reducing (+) conditions. STD, low molecular weight standards (BioRad, Hercules, CA): phosphorylase b, 97.4 kD; bovine serum albumin, 66.2 kD; ovalumin, 45.0 kD; carbonic anhydrase, 31.0; soybean trypsin inhibitor, 21.5 kD; lysozyme, 14.4 kD.

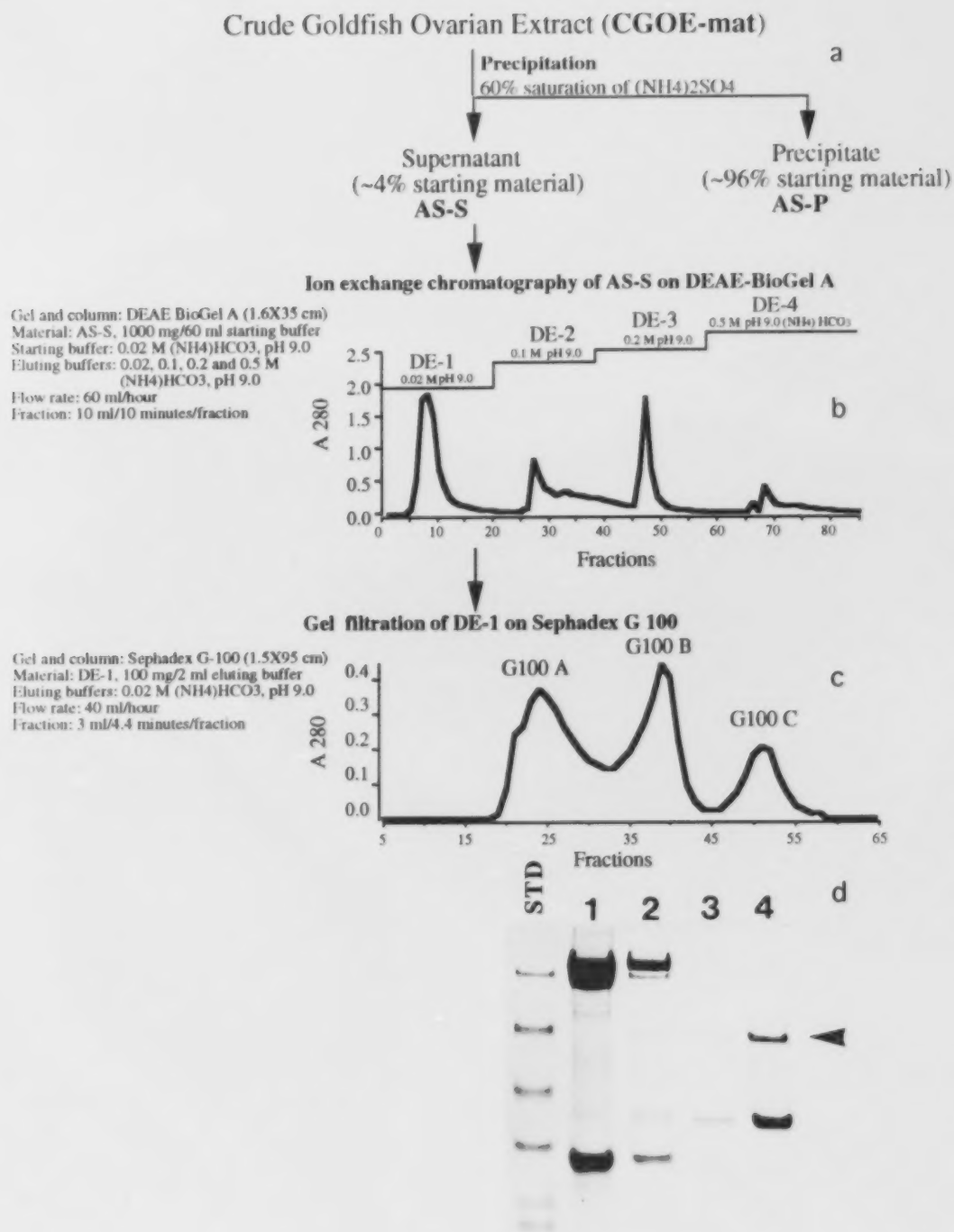


Fig. 9.2. Fractionation of crude goldfish ovarian extract (CGOE-mat) I. CGOE-mat was fractionated by precipitation (a), ion exchange (b) and gel filtration (c). The isolation steps were monitored by Weber and Osborn's SDS-PAGE (d); STD, low molecular weight standard; 1, CGOE-mat; 2, AS-S; 3, DE-1; 4, G 100 A.

Separation of G 100 A on DEAE-BioGel A

a

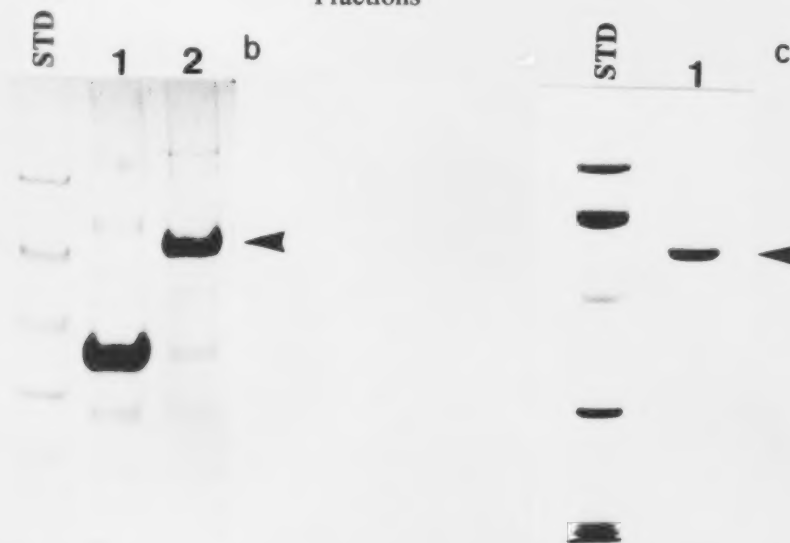
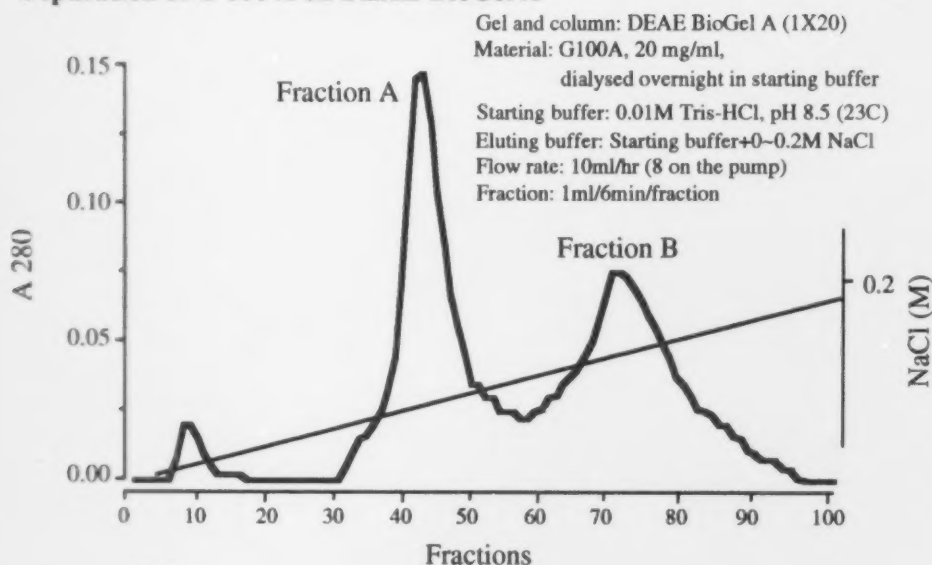


Fig. 9.3. Fractionation of crude goldfish ovarian extract (CGOE-mat) II. G 100 A from gel filtration was further fractionated by ion exchange (a) and the fractions A and B were analyzed by Weber and Osborn's SDS-PAGE (b); STD, low molecular standard; 1, Fraction A; 2, Fraction B. The final step of purification was preparative SDS electrophoresis and the purity was examined by analytical Laemmli's SDS-PAGE (c); STD, low molecular standard; 1, the purified protein of 57 kD.

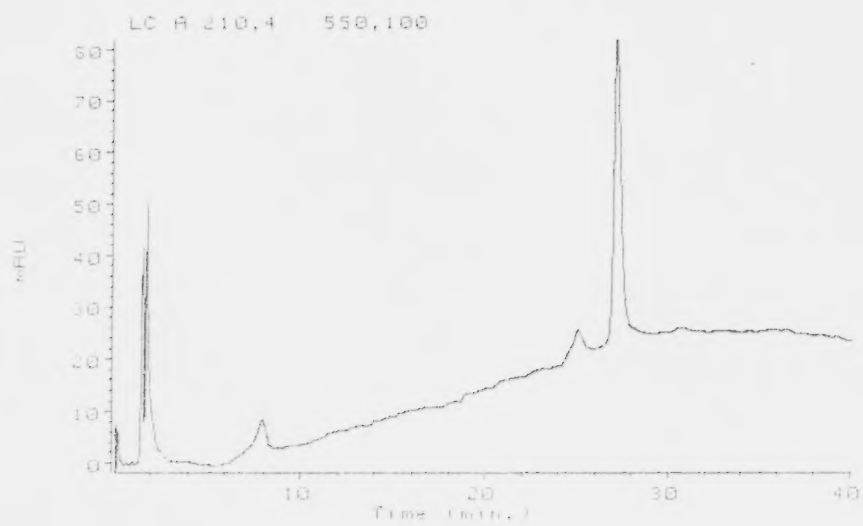


Fig. 9.4. Reversed-phase HPLC of the 57 kD protein purified by preparative SDS electrophoresis.

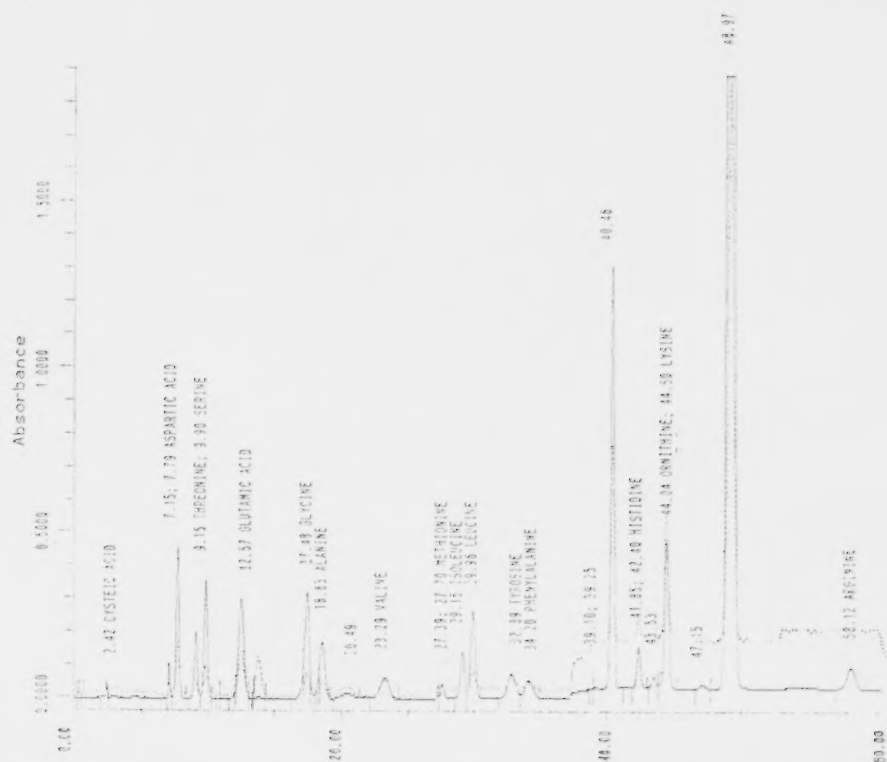


Fig. 9.5. Ion exchange chromatogram of goldfish transferrin hydrolysate

Table 9.1. Amino acid composition of the 70 kD goldfish transferrin (reduced) calculated from the chromatogram show above

Amino acid	Retention Time (min)	Concentration (nmol)	Residues/mole (estimation)
Aspartic acid	7.786	2.675	56.5
Threonine	9.150	1.364	29
Serine	9.900	1.882	40
Glutamic acid	12.570	3.321	70
Glycine	17.479	2.703	57
Alanine	18.628	1.755	37
Valine	23.289	1.140	24
Methionine	27.696	0.237	5
Isoleucine	29.152	0.941	20
Leucine	29.955	1.874	39.5
Tyrosine	32.886	0.943	20
Phenylalanine	34.204	0.700	15
Histidine	42.400	0.627	13.5
Lysine	44.496	2.338	49.5
Arginine	58.123	0.936	20
Proline	13.870	1.135	24

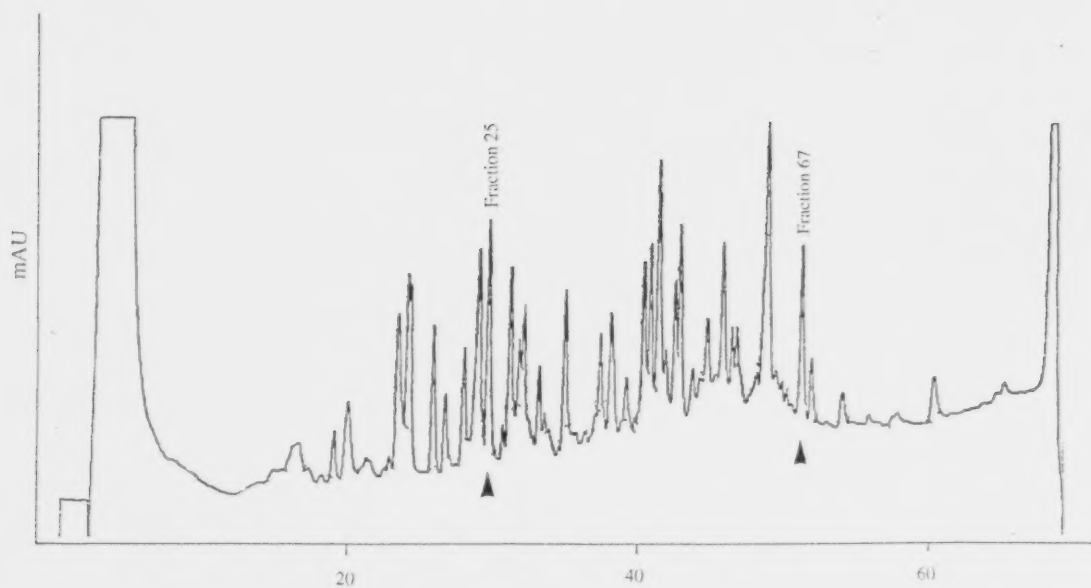


Fig. 9.6. Reversed-phase HPLC of the purified 57 kD protein digested with trypsin. Fraction 25 and 67 were chosen for determination of the amino acid sequences using automated Edman degradation.

N-terminal sequence:

Goldfish transferrin:	A Q(D,P) V K X(P) V K S Q N E L
	: : : : :
Toad transferrin:	E K Q V R W C V K S N S E L

Fraction 25 sequence:

Goldfish transferrin:	V P A H A V I T R
	: : : : : . : :
Toad transferrin:	V P A H A V L T R
(265-273)	

Fraction 67 sequence:

Goldfish transferrin:	(G) G D A D A I T V D A E H V Y L A G L
	: : : : : . : . : . : : : :
Toad transferrin:	K G D A D A V T L D G G Y M Y T A G L
(397-415)	

Fig. 9.7. Amino acid sequence alignment of three segments of goldfish ovarian transferrin with *Xenopus* transferrin (Moskaitis et al., 1990)

References

- Aisen, P. and Listowsky, I. 1980 Iron transport and storage proteins. *Ann Rev Biochem* 49:357-393
- Barnes, D. and Sato, G. 1980 Serum-free cell culture: a unifying approach. *Cell* 22:649-658
- Beach, R.L., Popiela, H. and Festoff, B.W. 1983 The identification of neurotrophic factor as a transferrin. *FEBS Lett* 156:151-156
- Eklblom, P., Thesleff, I., Saxen, L., Miettinen, A. and Timpl, R. 1983 Transferrin as a fetal growth factor: acquisition of responsiveness related to embryonic induction. *Proc Natl Acad Sci USA* 80:2651-2655
- Fischer, W.H., Karr, D., Jackson, B., Park, M. and Vale, W. 1991 Microsequence analysis of proteins purified by gel electrophoresis. *Methods in Neuroscience* 6:69-84
- Huggenvik, J.I., Idzerda, R.L., Haywood, L. Lee, D.C., McKnight, G.S. and Griswold, M.D.S. 1987 Transferrin messenger ribonucleic acid: molecular cloning and hormonal regulation in rat Sertoli cells. *Endocrinology* 120:332-340
- Khalfoun, B., Barthelemy, C., Crouzat-Reynes, C. and Bardos, P. 1986 A simple and sensitive solid-phase radioimmunoassay for measuring the transferrin content of human biological fluids: its application to seminal plasma. *Int J Biochem* 18:1135-1139
- Laemmli, U.K. 1970 Cleavage of structural proteins during assembly of the head of the bacteriophage T4. *Nature* 227:680-685
- Levin, M.J., Tuil, D., Uzan, G., Dreyfus, J-C. and Kahn, A. 1984 Expression of the transferrin gene during development of non-hepatic tissues: high level of transferrin mRNA in fetal muscle and adult brain. *Biochem Biophys Res Commun* 122:212-217
- Moskaitis, J.E., Pastori, R.L. and Schoenberg, D.R. 1990 The nucleotide sequence of *Xenopus laevis* transferrin mRNA. *Nucleic Acids Res* 18:6135-6135
- Ozols, J. 1990 Amino acid analysis. *Meth Enzymol* 182:587-601
- Roberts, K.P., Awoniyi, C.A., Santulli, R. and Zirkin, B.R. 1991 Regulation of Sertoli cell transferrin and sulfated glycoprotein-2 messenger ribonucleic acid levels during the restoration of spermatogenesis in the adult hypophysectomized rat. *Endocrinology* 129:3417-3423
- Roberts, K.P., Santulli, R., Seiden, J. and Zirkin, B.R. 1992 The effect of testosterone withdrawal and subsequent germ cell depletion on transferrin and sulfated glycoprotein-2 messenger ribonucleic acid levels in the adult rat testis. *Biol Reprod* 47:92-96

- Shivaji, S., Scheit, K-H. and Bhargava, P.M. 1990 Proteins of seminal plasma. John Wiley & Sons, Inc., New York, p. 421-425
- Skinner, M.K. and Griswold, M.D. 1980 Sertoli cells synthesize and secrete transferrin-like protein. *J Biol Chem* 255:9523-9525
- Sylvester, S.R. and Griswold, M.D. 1984 Localization of transferrin and transferrin receptors in rat testes. *Biol Reprod* 31:195-203
- Weber, K. and Osborn, M. 1969 The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J Biol Chem* 244:4406-4412